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(54) **CANAL CALCIQUE DE TYPE T**
(54) **T-TYPE CALCIUM CHANNEL**

(57)

The present invention is directed to isolated nucleic acid molecules encoding pancreatic T-type calcium channels and vectors and host cells comprising such. The invention is further directed to methods and compositions which modulate the expression of pancreatic T-type calcium channels, including antisense. An isolated pancreatic T-type calcium channel protein is provided, as well as antibodies directed to such protein. Pharmaceutical compositions and methods of treatment involving pancreatic T-type calcium channels are also provided.



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(54) Title: T-TYPE CALCIUM CHANNEL		
(57) Abstract <p>The present invention is directed to isolated nucleic acid molecules encoding pancreatic T-type calcium channels and vectors and host cells comprising such. The invention is further directed to methods and compositions which modulate the expression of pancreatic T-type calcium channels, including antisense. An isolated pancreatic T-type calcium channel protein is provided, as well as antibodies directed to such protein. Pharmaceutical compositions and methods of treatment involving pancreatic T-type calcium channels are also provided.</p>		

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T-TYPE CALCIUM CHANNEL

This application claims priority of U.S. Provisional Patent Application No. 60/098,004, filed August 26, 1998, and of U.S. Provisional Patent Application No. 60/117,399, filed January 27, 1999.

The subject matter of this application was made with support from the United States Government under National Institutes of Health Grant No. 5-20174. The U.S. Government may have certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates generally to calcium channel proteins, and more particularly to pancreatic T-type calcium channel proteins and uses thereof.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced, many in parenthesis. Full citations for each of these publications are provided at the end of the Detailed Description. The disclosures of each of these publications in their entireties are hereby incorporated by reference in this application.

Insulin secretion from pancreatic β -cells is the primary physiological mechanism of blood glucose regulation. A rise in blood glucose concentration stimulates release of insulin from the pancreas, which in turn promotes glucose uptake in peripheral tissues and consequently lowers blood glucose levels, reestablishing euglycemia. Non-insulin dependent diabetes mellitus (NIDDM) (type II diabetes) is associated with an impairment in glucose-induced insulin secretion in pancreatic β -cells (Vague and Moulin, 1982).

Voltage-gated Ca^{2+} channels mediate a rapidly activated inward movement of Ca^{2+} ions that underlies the

stimulation of insulin secretion in β -cells (Boyd III 1991). In different tissues, four types of Ca^{2+} channels have been described (L(P/Q), T, N, and E channels). The purified L-type Ca^{2+} channel consists of five subunits: α_1 ,
5 α_2 , β , γ , δ (Catterall 1991). The primary structure of the α_1 subunit is organized in four homologous domains containing six transmembrane segments (Catterall 1988).

Rat and human pancreatic β -cells are equipped with L-type and T-type Ca^{2+} channels (Hiriart and Matteson,
10 1988; Davalli et al., 1996). L-type Ca^{2+} channels, activated at high voltages and having large unitary conductance and dihydropyridine-sensitivity, are considered the major pipeline for Ca^{2+} influx into the β -cell (Keahey et al., 1989). In contrast, T-type calcium
15 channels activate at low voltages and have small unitary conductance and dihydropyridine-insensitivity.

The physiological function of T-type Ca^{2+} channels in β -cell insulin-secretion has been demonstrated (Bhattacharjee et al., 1997). These channels facilitate
20 exocytosis by enhancing electrical activity in these cells. L-type and T-type Ca^{2+} channels, under normal conditions, work in concert promoting the rise in $[\text{Ca}^{2+}]_i$ during glucose-stimulated insulin secretion. In β -cells, over-expressed T-type Ca^{2+} channels may be, at least in
25 part, responsible for the hyper-responsiveness of insulin secretion to non-glucose depolarizing stimuli in GK rat and in rat with NIDDM induced by neonatal injection of streptozotocin (Kato et al., 1994; Kato et al., 1996). However, over-expressed T-type calcium channels over time
30 will ultimately lead to an elevation of basal Ca^{2+} through its window current properties. Therefore, there is a dual effect of T-type Ca^{2+} channels in β -cells depending upon channel number and membrane potential.

Two isoforms of L-type Ca^{2+} channel $\alpha 1$ subunits have been identified in β -cells (Seino et al., 1992; Yaney et al., 1992). The rat neuronal T-type calcium channel has recently been cloned (Perez-Reyes et al., 1998). Other
5 subunits of T-type Ca^{2+} channel have yet to be identified.

Given the evidence that T-type calcium channels are associated with type II diabetes, a need exists to further characterize T-type calcium channels.

10

SUMMARY OF THE INVENTION

To this end, the subject invention provides an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel. The invention also provides an antisense nucleic acid molecule complementary to at least
15 a portion of the mRNA encoding the pancreatic T-type calcium channel.

The isolated nucleic acid molecules of the invention can be inserted into suitable expression vectors and/or host cells. Expression of the nucleic acid molecules
20 encoding the pancreatic T-type calcium channel results in production of pancreatic T-type calcium channel in a host cell. Expression of the antisense nucleic acid molecules in a host cell results in decreased expression of the pancreatic T-type calcium channel.

25 The invention further provides a ribozyme having a recognition sequence complementary to a portion of mRNA encoding a pancreatic T-type calcium channel. The ribozyme can be introduced into a cell to also achieve decreased expression of pancreatic T-type calcium channel
30 in the cell.

The invention further provides a method of screening a substance for the ability of the substance to modify T-type calcium channel function, and a method of obtaining DNA encoding a pancreatic T-type calcium channel.

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Further provided is an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel, wherein the nucleic acid molecule encodes a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence. The second amino acid sequence is as shown in SEQ ID NO:2.

The invention further provides a DNA oligomer capable of hybridizing to a nucleic acid molecule encoding a pancreatic T-type calcium channel. The DNA oligomer can be used in a method of detecting presence of a pancreatic T-type calcium channel in a sample, which method is also provided by the subject invention.

The invention also provides an isolated pancreatic T-type calcium channel protein, and antibodies or antibody fragments specific for the pancreatic T-type calcium channel protein. The antibodies and antibody fragments can be used to detect the presence of the pancreatic T-type calcium channel protein in samples. Further provided is an isolated pancreatic T-type calcium channel protein encoded by a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence, the second amino acid sequence as shown in SEQ ID NO:2.

The subject invention further provides a method of modifying insulin secretion by pancreatic beta cells, the method comprising modifying levels of functional T type calcium channels in the pancreatic beta cells. The invention further provides a method of treating type II diabetes in a subject, the method comprising administering to the subject an amount of a compound effective to modify levels of functional T type calcium channel in the pancreatic beta cells of the subject.

The invention also provides a method of modifying basal calcium levels in cells, a method of modifying the

action potential of L type calcium channels in cells, a method of modifying pancreatic beta cell death, a method of modifying pancreatic beta cell proliferation, and a method of modifying calcium influx through L type calcium channels in cells, each of the methods comprising modifying levels of functional T type calcium channels in the cells.

BRIEF DESCRIPTION OF THE DRAWINGS

10 These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Fig. 1A illustrates a comparison of the nucleotide sequence of α_1 G-INS (1) and α_1 G (2) at the 5'-end regions (aa1-67 of α_1 G). The four insertions are indicated with arrow heads. The capital ATG represents the start codon for each cDNA;

Fig. 1B is a schematic illustration representing partial rat genomic nucleotide composition between Domain III and IV. Genomic DNA contained an exon specific to α_1 G (shaded circle) and an exon specific to the α_1 subunit of T-type Ca^{2+} deduced from INS-1 (shaded rectangle) between 4845 and 5256 of the cDNA sequence. Other exons (open rectangles) are identical between the two cDNAs. The bold letters indicate the nucleotides coding Gly-1667;

Figs. 2A-2D illustrate expression of α_1 G-INS in *Xenopus* oocytes. Fig. 2A illustrates 40 mM Ca^{2+} currents elicited by depolarizing pulses from -60 to 40 mV. Fig. 2B illustrates time constants of activation and inactivation measured at test potentials between -30 and 30 mV. The time constants of activation were obtained by fitting the increasing portion (activation) of currents with the Hodgkin-Huxley equation where the m value was

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designated as four ($n = 6$). The time constants of inactivation were obtained by single exponential fitting ($n = 6$). Fig. 2C illustrates voltage-dependent conductance ($n = 7$) and Fig. 2D illustrates steady-state inactivation ($n = 3$) of expressed currents in oocytes. The holding potential for Figs. 2C and 2D was -80 mV. The currents in Fig. 2D were measured at -10 mV after varying 1000 ms pre-pulse potentials. Peak currents were normalized to the maximum current and then averaged (error bars represent SE);

Figs. 3A and 3B illustrate accumulative dose response relationships of the inhibitory effects of mibefradil on T- and L-type Ca^{2+} currents. Currents were measured with the whole-cell patch clamp configuration. Data from four experiments were normalized individually and then plotted as mean \pm standard error. Fig. 3A illustrates curve which was generated by fitting the data using one-to-one binding curve according to the equation $1/(1 + [\text{mibefradil}]/K_d)$. Fig. 3B is a dose response of L-type Ca^{2+} current obtained when perfusion of solutions containing different concentrations of mibefradil;

Fig. 4 illustrates reversibility of the inhibition of T and L-type currents by NiCl_2 and mibefradil, respectively. Open and solid circles represent the T-type Ca^{2+} current recorded before and after NiCl_2 (2 μl of 30 μM) and mibefradil (2 μl of 10 μM) were administrated, respectively. The open squares represent the L-type Ca^{2+} current recorded before and after mibefradil (2 μl of 10 μM) was administrated with perforated patch clamp configuration. The T-type Ca^{2+} current was measured at -30 mV with a holding potential of -80 mV with whole cell configuration. Arrow indicates the time when the drugs were delivered. $n = 3$ for each group experiments;

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Figs. 5A and 5B illustrate the long-term effect of mibefradil (10 nM) on L- and T- Ca^{2+} currents in the perforated-patch configuration. In Fig. 5A, solid and open circles represent the L-type Ca^{2+} current recorded in the cells with and without administration of mibefradil, respectively. Solid triangles represent T-type Ca^{2+} currents recorded in the cells after administering mibefradil. Mibefradil were delivered at time zero. $n = 4$ for each group experiments. In Fig. 5B, cells were cultured in medium with or without co-incubating 10 nM mibefradil for 2 hours. The current densities were recorded with perforated patch clamp configuration. $n = 14$ for each group experiments;

Fig. 6A illustrates accumulation of dm-mibefradil in the cells measured with mass spectrometry. The cells were first incubated with mibefradil (20 μM) for the duration indicated on the figure ($n = 3$). The inset (Fig. 6B) shows the primary data of mass spectrometry indicating peaks at 496 and 424, which correspond to mibefradil and dm-mibefradil, respectively;

Fig. 7A illustrates the effect of mibefradil and dm-mibefradil on L-type Ca^{2+} currents from inside cells. $n = 8$, *, $p < 0.01$ to the control;

Fig. 7B illustrates the effect of mibefradil or dm-mibefradil on T-type Ca^{2+} current from inside cells $n = 4$. All data were collected at 5 min after formation of whole cell patch. The pipette solution contained 1 μM of drug;

Fig. 8 illustrates basal $[\text{Ca}^{2+}]_i$ measured in an INS-1 cell. T-type calcium channel antagonist mibefradil (1 μM) reduced basal $[\text{Ca}^{2+}]_i$ in a single cell in the bath solution without glucose. The $[\text{Ca}^{2+}]_i$ was measured with the emission ratio of Fura-2 AM (F380/F340) then

calibrated with the standard solution purchased from Molecular Probes Inc. (OR);

Fig. 9A illustrates that intracellular perfusion of a solution containing 272 nM free calcium concentration inhibits the L-type calcium current. Currents were elicited by a step voltage to +10 mV, with holding potential of -80 mV;

Fig. 9B illustrates the effect of perfusing in high calcium concentration on the IV calcium current relationship. Closed circles represent the cell before perfusion, and open circles represent perfusion of 272 nM free calcium;

Fig. 9C illustrates the effect of intracellular perfusion of different calcium concentrations on L-type calcium current over time. Squares represent perfusion from high calcium to low calcium (intracellular solution contained 632 nM then perfused by a solution with 10 mM EGTA), triangles represent perfusion from low calcium to 272 nM calcium, and circles represent low calcium to 632 nM calcium;

Fig. 9D illustrates the effect of high calcium on the T-type calcium channel current. Tail currents were elicited by a voltage step to -30 mV for 10 ms;

Fig. 10 illustrates that reestablishment of basal calcium causes stereotyped calcium influx. A cell was twice perfused with 50 mM KCl with an intervening perfusion of the original bath solution to restore membrane potential;

Fig. 11 illustrates that elevated basal Ca^{2+} causes a defect in the Ca^{2+} transient. A cell was twice perfused with 50 mM KCl with an intervening perfusion of the original bath solution to restore membrane potential. The second perfusion occurred prior to reestablishment of the original basal $[\text{Ca}^{2+}]_i$ of about 60 nM;

Fig. 12 illustrates a model for glucose-stimulated insulin release;

Fig. 13 illustrates that mibefradil ($1\ \mu\text{M}$) blocks T- and L-type Ca^{2+} current in INS-1 cells. The relative
5 current of T type Ca channel is obtained by measuring their slow deactivated tail current ($n = 8$);

Fig. 14 illustrates that mibefradil and NiCl_2 reversibly block T type Ca^{2+} current in INS-1 cells. Drugs were administered into the recording chamber at 180
10 seconds from the beginning of recording. $N = 3$;

Fig. 15 illustrates the activation and inactivation curves for INS-1 cells, revealing a "window current";

Fig. 16 illustrates the effect of NiCl_2 , mibefradil, and nifedipine on basal insulin secretion in NIT-1 cells.
15 The glucose concentration is 3 mM in the experiments;

Fig. 17 illustrates that the T type calcium channel antagonist NiCl_2 ($30\ \mu\text{M}$) reduced the frequency of transient spontaneous elevation of $[\text{Ca}^{2+}]_i$ in a single cell in the bath solution without glucose;

20 Fig. 18 illustrates the effect of 30 mM NiCl_2 on the $[\text{Ca}^{2+}]_i$ under non-stimulus conditions. Data was collected from the cells with "high" initial basal $[\text{Ca}^{2+}]_i$ (about 100 nM). $n = 13$;

Figs. 19A and 19B illustrate that hyperpolarization
25 induced an increase in number of action potentials and a decrease in onset latencies. $N = 40$;

Figs. 20A and 20B illustrate the dose-dependent effect of NiCl_2 on insulin secretion. Cells were placed in a medium containing 11.1 mM glucose and a decrease in
30 onset latencies. $N = 40$;

Fig. 21 illustrates "run-up" in whole cell recording;

Fig. 22 illustrates KCl induced Ca^{2+} influx in the INS-1 cells treated with streptozotocin. $n = 13$;

Fig. 23A-23F illustrate the results of cytokine treatment. LVA Ca^{2+} currents were induced by cytokine treatment (IL- 1β , 25 U/ml; IFN γ , 300 U/ml) for 6 h in primary cultured mouse islet cells, but not in α -TC1 cells. An LVA current was elicited by a -40 mV test pulse in an islet cell (Fig. 23A), but the same current was not detected in α -TC1 cells (Fig. 23C). The Ca^{2+} current density-voltage relationships obtained from islet cells (Fig. 23B) and α -TC1 cells (Fig. 23D) with and without cytokine treatment are shown. The open circles represent the current densities of untreated cells ($n = 10$ for islet cells; $n = 20$ for α -TC1 cells), and the filled circles represent the current densities of cells treated by cytokines ($n = 21$ for islet cells; $n = 21$ for α -TC1 cells). The recordings were elicited by voltages ranging from -50 to +20 mV for 100 msec. All experiments were performed at -80 mV. Fig. 23E shows steady state inactivation of LVA tail currents elicited by a 10-msec depolarizing (-10 mV) pulse followed by a 50-msec hyperpolarizing pulse (-100 mV), with a holding potential of -80 mV. Fig. 23F shows that NiCl_2 (10 μM) blocked the cytokine induced LVA Ca^{2+} current elicited at a -30 mV step pulse in an islet cell;

Figs. 24A and 24B illustrate the effects of cytokines on $[\text{Ca}^{2+}]_i$ in mouse islet cells and α -TC1 cells. In Fig. 24A, basal $[\text{Ca}^{2+}]_i$ of primary cultured mouse islet cells was approximately 3-fold higher after cytokine treatment. NiCl_2 (10 μM), but not nifedipine (10 μM), prevented the increase in $[\text{Ca}^{2+}]_i$. In Fig. 24B, basal $[\text{Ca}^{2+}]_i$ in α -TC1 cells was unaffected by cytokine treatment. Cytokine treatment consisted of IL- 1β (25 U/ml) and IFN γ (300 U/ml) for 6 h; and

Figs. 25A and 25B illustrate the effects of NiCl_2 on cytokine-induced β -TC3 cell death. NiCl_2 (20 μM)

significantly reduced cell death induced by cytokines in both a time (Fig. 25A) and dose-dependent (Fig. 25B) manner ($n = 3$). Cytokine treatment consisted of IL-1 β (25 U/ml), IFN γ (100 U/ml), and TNF α (100 U/ml) in Fig. 25A and of IL-1 β (25 U/ml), TNF α (100 U/ml), and various concentrations of IFN γ as indicated in Fig. 25A. The first dose, 0, represents zero concentration for all three cytokines. The concentration of nifedipine was 10 μ M in both Fig. 25A and Fig. 25B.

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DETAILED DESCRIPTION OF THE INVENTION

The term "nucleic acid", as used herein, refers to either DNA or RNA. "Nucleic acid sequence" or "polynucleotide sequence" refers to a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA, and nonfunctional DNA or RNA.

"Isolated" nucleic acid refers to nucleic acid which has been separated from an organism in a substantially purified form (i.e. substantially free of other substances originating from that organism), and to synthetic nucleic acid.

By a nucleic acid sequence "homologous to" or "complementary to", it is meant a nucleic acid that selectively hybridizes, duplexes or binds to DNA sequences encoding the protein (channel) or portions thereof when the DNA sequences encoding the protein are present in a human genomic or cDNA library. A DNA sequence which is similar or complementary to a target sequence can include sequences which are shorter or longer than the target sequence so long as they meet the functional test set forth.

Typically, the hybridization is done in a Southern blot protocol using a 0.2X SSC, 0.1% SDS, 65°C wash. The term "SSC" refers to a citrate-saline solution of 0.15M sodium chloride and 20 mM sodium citrate. Solutions are
5 often expressed as multiples or fractions of this concentration. For example, 6X SSC refers to a solution having a sodium chloride and sodium citrate concentration of 6 times this amount or 0.9 M sodium chloride and 120 mM sodium citrate. 0.2X SSC refers to a solution 0.2
10 times the SSC concentration or 0.03M sodium chloride and 4 mM sodium citrate.

The phrase "nucleic acid molecule encoding" refers to a nucleic acid molecule which directs the expression of a specific protein or peptide. The nucleic acid
15 sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein or peptide. The nucleic acid molecule includes both the full length nucleic acid sequences as well as non-full length sequences derived
20 from the full length protein. It being further understood that the sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell.

25 The term "located upstream" as used herein refers to linkage of a promoter upstream from a nucleic acid (DNA) sequence such that the promoter mediates transcription of the nucleic acid (DNA) sequence.

The term "vector", refers to viral expression
30 systems, autonomous self-replicating circular DNA (plasmids), and includes both expression and nonexpression plasmids. Where a recombinant microorganism or cell is described as hosting an "expression vector," this includes both extrachromosomal

circular DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous
5 structure, or the vector may be incorporated within the — host's genome.

The term "plasmid" refers to an autonomous circular DNA molecule capable of replication in a cell, and includes both the expression and nonexpression types.
10 Where a recombinant microorganism or cell is described as hosting an "expression plasmid", this includes latent viral DNA integrated into the host chromosome(s). Where a plasmid is being maintained by a host cell, the plasmid is either being stably replicated by the cell during
15 mitosis as an autonomous structure, or the plasmid is incorporated within the host's genome.

The phrase "heterologous protein" or "recombinantly produced heterologous protein" refers to a peptide or protein of interest produced using cells that do not have
20 an endogenous copy of DNA able to express the peptide or protein of interest. The cells produce the peptide or protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequences. The recombinant peptide or protein will not be found in
25 association with peptides or proteins and other subcellular components normally associated with the cells producing the peptide or protein.

The following terms are used to describe the sequence relationships between two or more nucleic acid
30 molecules or polynucleotides, or between two or more amino acid sequences of peptides or proteins: "reference sequence", "comparison window", "sequence identity", "sequence homology", "percentage of sequence identity", "percentage of sequence homology", "substantial

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identity", and "substantial homology". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing or may comprise a complete cDNA or gene sequence.

Optimal alignment of sequences for aligning a comparison window may be conducted, for example, by the local homology algorithm of Smith and Waterman (1981), by the homology alignment algorithm of Needleman and Wunsch (1970), by the search for similarity method of Pearson and Lipman (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.).

As applied to nucleic acid molecules or polynucleotides, the terms "substantial identity" or "substantial sequence identity" mean that two nucleic acid sequences, when optimally aligned (see above), share at least 90 percent sequence identity, preferably at least 95 percent sequence identity, more preferably at least 96, 97, 98 or 99 percent sequence identity.

"Percentage nucleotide (or nucleic acid) identity" or "percentage nucleotide (or nucleic acid) sequence identity" refers to a comparison of the nucleotides of two nucleic acid molecules which, when optimally aligned, have approximately the designated percentage of the same nucleotides. For example, "95% nucleotide identity" refers to a comparison of the nucleotides of two nucleic acid molecules which when optimally aligned have 95% nucleotide identity. Preferably, nucleotide positions which are not identical differ by redundant nucleotide substitutions (the nucleotide substitution does not change the amino acid encoded by the particular codon).

As further applied to nucleic acid molecules or polynucleotides, the terms "substantial homology" or "substantial sequence homology" mean that two nucleic acid sequences, when optimally aligned (see above), share at least 90 percent sequence homology, preferably at least 95 percent sequence homology, more preferably at least 96, 97, 98 or 99 percent sequence homology.

"Percentage nucleotide (or nucleic acid) homology" or "percentage nucleotide (or nucleic acid) sequence homology" refers to a comparison of the nucleotides of two nucleic acid molecules which, when optimally aligned, have approximately the designated percentage of the same nucleotides or nucleotides which are not identical but differ by redundant nucleotide substitutions (the nucleotide substitution does not change the amino acid encoded by the particular codon). For example, "95% nucleotide homology" refers to a comparison of the nucleotides of two nucleic acid molecules which when optimally aligned have 95% nucleotide homology.

As applied to polypeptides, the terms "substantial identity" or "substantial sequence identity" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap, share at least 90 percent sequence identity, preferably at least 95 percent sequence identity, more preferably at least 96, 97, 98 or 99 percent sequence identity.

"Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity. Preferably, residue positions which

are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to affect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

As further applied to polypeptides, the terms "substantial homology" or "substantial sequence homology" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap, share at least 90 percent sequence homology, preferably at least 95 percent sequence homology, more preferably at least 96, 97, 98 or 99 percent sequence homology.

"Percentage amino acid homology" or "percentage amino acid sequence homology" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids or conservatively substituted amino acids. For example, "95% amino acid homology" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid homology. As used herein, homology refers to identical amino acids or residue positions which are not identical but differ only by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to affect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The phrase "substantially purified" or "isolated" when referring to a protein (or peptide), means a chemical composition which is essentially free of other cellular components. It is preferably in a homogeneous state although it can be in either a dry or aqueous

solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein (or peptide) which is the predominant species present in a preparation is substantially purified. Generally, a substantially purified or isolated protein (or peptide) will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein (or peptide) is purified to represent greater than 90% of all macromolecular species present. More preferably the protein (or peptide) is purified to greater than 95%, and most preferably the protein (or peptide) is purified to essential homogeneity, wherein other macromolecular species are not detected by conventional techniques. A "substantially purified" or "isolated" protein (or peptide) can be separated from an organism, synthetically or chemically produced, or recombinantly produced.

"Biological sample" or "sample" as used herein refers to any sample obtained from a living organism or from an organism that has died. Examples of biological samples include body fluids and tissue specimens.

High stringent hybridization conditions are selected at about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents,

i.e. salt or formamide concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. High stringency may be attained, for example, by overnight
5 hybridization at about 68°C in a 6X SSC solution, washing— at room temperature with 6X SSC solution, followed by washing at about 68°C in a 6X SSC solution then in a 0.6X SSX solution.

Hybridization with moderate stringency may be
10 attained, for example, by: 1) filter pre-hybridizing and hybridizing with a solution of 3X sodium chloride, sodium citrate (SSC), 50% formamide, 0.1M Tris buffer at pH 7.5, 5X Denhardt's solution; 2) pre-hybridization at 37°C for 4 hours; 3) hybridization at 37°C with amount of labeled
15 probe equal to 3,000,000 cpm total for 16 hours; 4) wash in 2X SSC and 0.1% SDS solution; 5) wash 4X for 1 minute each at room temperature and 4X at 60°C for 30 minutes each; and 6) dry and expose to film.

The phrase "selectively hybridizing to" refers to a
20 nucleic acid molecule that hybridizes, duplexes or binds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. By selectively hybridizing it is meant that a nucleic acid molecule binds to a given
25 target in a manner that is detectable in a different manner from non-target sequence under moderate, or more preferably under high, stringency conditions of hybridization. "Complementary" or "target" nucleic acid sequences refer to those nucleic acid sequences which
30 selectively hybridize to a nucleic acid molecule. Proper annealing conditions depend, for example, upon a nucleic acid molecule's length, base composition, and the number of mismatches and their position on the molecule, and must often be determined empirically. For discussions of

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nucleic acid molecule (probe) design and annealing conditions, see, for example, Sambrook et al. 1989.

It will be readily understood by those skilled in the art and it is intended here, that when reference is made to particular sequence listings, such reference includes sequences which substantially correspond to its complementary sequence and those described including allowances for minor sequencing errors, single base changes, deletions, substitutions and the like, such that any such sequence variation corresponds to the nucleic acid sequence of the signal peptide or other peptide/protein to which the relevant sequence listing relates.

The DNA molecules of the subject invention also include DNA molecules coding for protein analogs, fragments or derivatives of the protein which differ from naturally-occurring forms (the naturally-occurring protein) in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues, and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the protein) and which share the signal property of the naturally-occurring form. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

As used herein, a "peptide" refers to an amino acid sequence of three to one hundred amino acids, and

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therefore an isolated peptide that comprises an amino acid sequence is not intended to cover amino acid sequences of greater than 100 amino acids. Preferably, the peptides that can be identified and used in accordance with the subject invention (whether they be mimotope or anti-mimotope peptides) are less than 50 amino acids in length, and more preferably the peptides are five to 20 amino acids in length or 20-40 amino acids in length.

10 The peptides can contain any naturally-occurring or non-naturally-occurring amino acids, including the D-form of the amino acids, amino acid derivatives and amino acid mimics, so long as the desired function and activity of the peptide is maintained. The choice of including an (L)- or a (D)-amino acid in the peptides depends, in part, on the desired characteristics of the peptide. For example, the incorporation of one or more (D)-amino acids can confer increased stability on the peptide and can allow a peptide to remain active in the body for an extended period of time. The incorporation of one or more (D)-amino acids can also increase or decrease the pharmacological activity of the peptide.

The peptides may also be cyclized, since cyclization may provide the peptides with superior properties over their linear counterparts.

As used herein, the terms "amino acid mimic" and "mimetic" mean an amino acid analog or non-amino acid moiety that has the same or similar functional characteristic of a given amino acid. For instance, an amino acid mimic of a hydrophobic amino acid is one which is non-polar and retains hydrophobicity, generally by way of containing an aliphatic chemical group. By way of further example, an arginine mimic can be an analog of arginine which contains a side chain having a positive

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charge at physiological pH, as is characteristic of the guanidinium side chain reactive group of arginine.

In addition, modifications to the peptide backbone and peptide bonds thereof are also encompassed within the scope of amino acid mimic or mimetic. Such modifications—
5 can be made to the amino acid, derivative thereof, non-amino acid moiety or the peptide either before or after the amino acid, derivative thereof or non-amino acid moiety is incorporated into the peptide. What is
10 critical is that such modifications mimic the peptide backbone and bonds which make up the same and have substantially the same spacial arrangement and distance as is typical for traditional peptide bonds and backbones. An example of one such modification is the
15 reduction of the carbonyl(s) of the amide peptide backbone to an amine. A number of reagents are available and well known for the reduction of amides to amines such as those disclosed in Wann et al., JOC, 46:257 (1981) and Raucher et al., Tetrahedron. Lett., 21:14061 (1980). An
20 amino acid mimic is, therefor, an organic molecule that retains the similar amino acid pharmacophore groups as is present in the corresponding amino acid and which exhibits substantially the same spatial arrangement between functional groups.

25 The substitution of amino acids by non-naturally occurring amino acids and amino acid mimics as described above can enhance the overall activity or properties of an individual peptide based on the modifications to the backbone or side chain functionalities. For example,
30 these types of alterations to the specifically described amino acid substituents and exemplified peptides can enhance the peptide's stability to enzymatic breakdown and increase biological activity. Modifications to the

peptide backbone similarly can add stability and enhance activity.

One skilled in the art, using the above sequences or formulae, can easily synthesize the peptides. Standard
5 procedures for preparing synthetic peptides are well known in the art. The novel peptides can be synthesized using: the solid phase peptide synthesis (SPPS) method of Merrifield (J. Am. Chem. Soc., 85:2149 (1964)) or modifications of SPPS; or, the peptides can be
10 synthesized using standard solution methods well known in the art (see, for example, Bodanzsky, M., Principles of Peptide Synthesis, 2nd revised ed., Springer-Verlag (1988 and 1993)). Alternatively, simultaneous multiple peptide synthesis (SMPS) techniques well known in the art can be
15 used. Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, Calif.) or using the manual peptide synthesis technique described by Houghten, Proc.
20 Natl. Acad. Sci., USA 82:5131 (1985).

With these definitions in mind, the subject invention provides an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel. The nucleic acid molecule can be deoxyribonucleic acid (DNA)
25 or ribonucleic acid (RNA, including messenger RNA or mRNA), genomic or recombinant, biologically isolated or synthetic.

The DNA molecule can be a cDNA molecule, which is a DNA copy of a messenger RNA (mRNA) encoding the channel.

30 An example of such a pancreatic T-type calcium channel is the rat pancreatic T-type calcium channel encoded by the nucleotide sequence as shown in SEQ ID NO:1. The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO:2.

The invention also provides an antisense nucleic acid molecule that is complementary to at least a portion of the mRNA encoding the pancreatic T-type calcium channel. Antisense nucleic acid molecules can be RNA or
5 single-stranded DNA, and can be complementary to the entire mRNA molecule encoding the channel (i.e. of the same nucleotide length as the entire molecule). It may be desirable, however, to work with a shorter molecule. In this instance, the antisense molecule can be
10 complementary to a portion of the entire mRNA molecule encoding the channel. These shorter antisense molecules are capable of hybridizing to the mRNA encoding the entire molecule, and preferably consist of about twenty to about one hundred nucleotides. These antisense
15 molecules can be used to reduce levels of pancreatic T-type calcium channel, by introducing into cells an RNA or single-stranded DNA molecule that is complementary to at least a portion of the mRNA of the channel (i.e. by introducing an antisense molecule). The antisense
20 molecule can base-pair with the mRNA of the channel, preventing translation of the mRNA into protein. Thus, an antisense molecule to the channel can prevent translation of mRNA encoding the channel into a functional channel protein. It may be desirable to place
25 the antisense molecule downstream and under the control of the insulin promoter, so that the antisense will prevent translation of mRNA encoding the T type calcium channel only in islet cells of the pancreas (not affecting brain or heart T type calcium channels). It
30 should also be apparent that 100% prevention of T type calcium channel is not desirable, since a minimal basal Ca^{2+} level is required to be maintained by the T type calcium channel.

More particularly, an antisense molecule complementary to at least a portion of mRNA encoding a pancreatic T-type calcium channel can be used to decrease expression of a functional channel. A cell with a first
5 level of expression of a functional pancreatic T-type calcium channel is selected, and then the antisense molecule is introduced into the cell. The antisense molecule blocks expression of functional pancreatic T-type calcium channel, resulting in a second level of
10 expression of a functional pancreatic T-type calcium channel in the cell. The second level is less than the initial first level.

Antisense molecules can be introduced into cells by any suitable means. In one embodiment, the antisense RNA
15 molecule is injected directly into the cellular cytoplasm, where the RNA interferes with translation. A vector may also be used for introduction of the antisense molecule into a cell. Such vectors include various plasmid and viral vectors. For a general discussion of
20 antisense molecules and their use, see Han et al. 1991 and Rossi 1995.

The invention further provides a special category of antisense RNA molecules, known as ribozymes, having recognition sequences complementary to specific regions
25 of the mRNA encoding the pancreatic T-type calcium channel. Ribozymes not only complex with target sequences via complementary antisense sequences but also catalyze the hydrolysis, or cleavage, of the template mRNA molecule. Examples, which are not intended to be
30 limiting, of suitable regions of the mRNA template to be targeted by ribozymes are any of the homologous regions identified by comparing the various T-type calcium channels, and particularly pancreatic β -cell T-type channels.

Expression of a ribozyme in a cell can inhibit gene expression (such as the expression of a pancreatic T-type calcium channel). More particularly, a ribozyme having a recognition sequence complementary to a region of a mRNA
5 encoding a pancreatic T-type calcium channel can be used — to decrease expression of pancreatic T-type calcium channel. A cell with a first level of expression of pancreatic T-type calcium channel is selected, and then the ribozyme is introduced into the cell. The ribozyme
10 in the cell decreases expression of pancreatic T-type calcium channel in the cell, because mRNA encoding the pancreatic T-type calcium channel is cleaved and cannot be translated.

Ribozymes can be introduced into cells by any
15 suitable means. In one embodiment, the ribozyme is injected directly into the cellular cytoplasm, where the ribozyme cleaves the mRNA and thereby interferes with translation. A vector may be used for introduction of the ribozyme into a cell. Such vectors include various
20 plasmid and viral vectors (note that the DNA encoding the ribozyme does not need to be "incorporated" into the genome of the host cell; it could be expressed in a host cell infected by a viral vector, with the vector expressing the ribozyme, for instance). For a general
25 discussion of ribozymes and their use, see Sarver et al. 1990, Chrissey et al. 1991, Rossi et al. 1992, and Christoffersen et al. 1995.

The nucleic acid molecules of the subject invention can be expressed in suitable host cells using
30 conventional techniques. Any suitable host and/or vector system can be used to express the pancreatic T-type calcium channel. For in vitro expression, Xenopus oocytes are preferred. For in vivo expression, the most suitable host cell is a pancreatic β -cell.

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Techniques for introducing the nucleic acid molecules into the host cells may involve the use of expression vectors which comprise the nucleic acid molecules. These expression vectors (such as plasmids and viruses; viruses including bacteriophage) can then be used to introduce the nucleic acid molecules into suitable host cells. For example, DNA encoding the pancreatic T-type calcium channel can be injected into the nucleus of a host cell or transformed into the host cell using a suitable vector, or mRNA encoding the pancreatic T-type calcium channel can be injected directly into the host cell, in order to obtain expression of pancreatic T-type calcium channel in the host cell.

Various methods are known in the art for introducing nucleic acid molecules into host cells. One method is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles (or RNA is injected directly into the cytoplasm of cells). Alternatively, DNA can be incubated with an inert carbohydrate polymer (dextran) to which a positively charged chemical group (DEAE, for diethylaminoethyl) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles stick in turn to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell. In another method, cells efficiently take in DNA in the form of a precipitate with calcium phosphate. In electroporation, cells are placed in a solution containing DNA and subjected to a brief electrical pulse that causes holes to open transiently in

their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium phosphate procedures. DNA can also be incorporated into
5 artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, DNA is absorbed to the surface of tungsten microprojectiles and fired into cells with a device resembling a shotgun.

10 Several of these methods, microinjection, electroporation, and liposome fusion, have been adapted to introduce proteins into cells. For review, see Mannino and Gould-Fogerite 1988, Shigekawa and Dower 1988, Capecchi 1980, and Klein et al. 1987.

15 Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. One such virus widely used for protein production is an insect virus, baculovirus. For a review of baculovirus vectors, see Miller (1989). Various viral vectors have
20 also been used to transform mammalian cells, such as bacteriophage, vaccinia virus, adenovirus, and retrovirus.

As indicated, some of these methods of transforming a cell require the use of an intermediate plasmid vector.
25 U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and
30 replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector using standard cloning procedures known in the art, as described by Sambrook et al. (1989).

Host cells into which the nucleic acid encoding the pancreatic T-type calcium channel has been introduced can be used to produce (i.e. to functionally express) the pancreatic T-type calcium channel. The function of the encoded pancreatic T-type calcium channel can be assayed — according to methods known in the art (Wang et al. 1996).

Having identified the nucleic acid molecules encoding pancreatic T-type calcium channels and methods for expressing the pancreatic T-type calcium channels encoded thereby, the invention further provides a method of screening a substance (for example, a compound or inhibitor) for the ability of the substance to modify T-type calcium channel function. The method comprises introducing a nucleic acid molecule encoding the pancreatic T-type calcium channel into a host cell, and expressing the pancreatic T-type calcium channel encoded by the molecule in the host cell. The cell is then exposed to a substance and evaluated to determine if the substance modifies the function of the T-type calcium channel. From this evaluation, substances effective in altering the function of the T-type calcium channel can be found. Such agents may be, for example, calcium channel inhibitors, agonists, or antagonists (for example, mibefradil and mibefradil analogues, amiloride, NiCl_2 , antisense molecules, and second messengers).

The evaluation of the cell to determine if the substance modifies the function of the T-type calcium channel can be by any means known in the art. The evaluation can comprise the direct monitoring of expression of T-type calcium channel in the host cell, or the evaluation can be indirect and comprise the monitoring of calcium transport by the channel (such as by the methods disclosed by Wang et al. 1996).

The nucleic acid molecules of the subject invention can be used either as probes or for the design of primers to obtain DNA encoding other pancreatic T-type calcium channels by either cloning and colony/plaque
5 hybridization or amplification using the polymerase chain-reaction (PCR).

Specific probes derived from SEQ ID NO:1 can be employed to identify colonies or plaques containing cloned DNA encoding a member of the pancreatic T-type
10 calcium channel family using known methods (see Sambrook et al. 1989). One skilled in the art will recognize that by employing such probes under high stringency conditions (for example, hybridization at 42°C with 5X SSPE and 50% formamide, washing at 50-65°C with 0.5X SSPE), sequences
15 having regions which are greater than 90% homologous or identical to the probe can be obtained. Sequences with lower percent homology or identity to the probe, which also encode pancreatic T-type calcium channels, can be obtained by lowering the stringency of hybridization and
20 washing (e.g., by reducing the hybridization and wash temperatures or reducing the amount of formamide employed).

More particularly, in one embodiment, the method comprises selection of a DNA molecule encoding a
25 pancreatic T-type calcium channel, or a fragment thereof, the DNA molecule having a nucleotide sequence as shown in SEQ ID NO:1, and designing an oligonucleotide probe for pancreatic T-type calcium channel based on SEQ ID NO:1. A genomic or cDNA library of an organism is then probed
30 with the oligonucleotide probe, and clones are obtained from the library that are recognized by the oligonucleotide probe so as to obtain DNA encoding another pancreatic T-type calcium channel.

Specific primers derived from SEQ ID NO:1 can be used in PCR to amplify a DNA sequence encoding a member of the pancreatic T-type calcium channel family using known methods (see Innis et al. 1990). One skilled in the art will recognize that by employing such primers under high stringency conditions (for example, annealing at 50-60°C, depending on the length and specific nucleotide content of the primers employed), sequences having regions greater than 75% homologous or identical to the primers will be amplified.

More particularly, in a further embodiment the method comprises selection of a DNA molecule encoding pancreatic T-type calcium channel, or a fragment thereof, the DNA molecule having a nucleotide sequence as shown in SEQ ID NO:1, designing degenerate oligonucleotide primers based on regions of SEQ ID NO:1, and employing such primers in the polymerase chain reaction using as a template a DNA sample to be screened for the presence of pancreatic T-type calcium channel-encoding sequences. The resulting PCR products can be isolated and sequenced to identify DNA fragments that encode polypeptide sequences corresponding to the targeted region of pancreatic T-type calcium channel.

Various modifications of the nucleic acid and amino acid sequences disclosed herein are covered by the subject invention. These varied sequences still encode a functional pancreatic T-type calcium channel. The invention thus further provides an isolated nucleic acid molecule encoding a pancreatic T-type calcium channel, the nucleic acid molecule encoding a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence, the second amino acid sequence as shown in SEQ ID NO:2. In further embodiments, the first amino acid sequence has at least

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95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO:2.

The invention further provides an isolated DNA oligomer capable of hybridizing to the nucleic acid molecule encoding pancreatic T-type calcium channel according to the subject invention. Such oligomers can be used as probes in a method of detecting the presence of pancreatic T-type calcium channel in a sample. More particularly, a sample can be contacted with the DNA oligomer and the DNA oligomer will hybridize to any pancreatic T-type calcium channel present in the sample, forming a complex therewith. The complex can then be detected, thereby detecting presence of pancreatic T-type calcium channel in the sample.

The complex can be detected using methods known in the art. Preferably, the DNA oligomer is labeled with a detectable marker so that detection of the marker after the DNA oligomer hybridizes to any pancreatic T-type calcium channel in the sample (wherein non-hybridized DNA oligomer has been washed away) is detection of the complex. Detection of the complex indicates the presence of pancreatic T-type calcium channel in the sample. As will be readily apparent to those skilled in the art, such a method could also be used quantitatively to assess the amount of pancreatic T-type calcium channel in a sample.

For detection, the oligomers can be labeled with, for example, a radioactive isotope, biotin, an element opaque to X-rays, or a paramagnetic ion. Radioactive isotopes are commonly used and are well known to those skilled in the art. Representative examples include indium-111, technetium-99m, and iodine-123. Biotin is a standard label which would allow detection of the biotin labeled oligomer with avidin. Paramagnetic ions are also

commonly used and include, for example, chelated metal ions of chromium (III), manganese (II), and iron (III). When using such labels, the labeled DNA oligomer can be imaged using methods known to those skilled in the art.

5 Such imaging methods include, but are not limited to, X-ray, CAT scan, PET scan, NMRI, and fluoroscopy. Other suitable labels include enzymatic labels (horseradish peroxidase, alkaline phosphatase, etc.) and fluorescent labels (such as FITC or rhodamine, etc.).

10 The invention further provides an isolated pancreatic T-type calcium channel protein. The protein is preferably encoded by a nucleotide sequence as shown in SEQ ID NO:1. The protein preferably has an amino acid sequence as shown in SEQ ID NO:2. Further provided is an
15 isolated pancreatic T-type calcium channel protein encoded by a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence, the second amino acid sequence as shown in SEQ ID NO:2. In further embodiments, the first amino acid sequence has
20 at least 95%, 96%, 97%, 98%, or 99% amino acid identity to SEQ ID NO:2.

The pancreatic T-type calcium channel molecule of the subject invention can include a leader sequence for targeting of the pancreatic T-type calcium channel
25 protein to the desired part of a cell.

It should be readily apparent to those skilled in the art that a met residue may need to be added to the amino terminal of the amino acid sequence of the mature pancreatic T-type calcium channel protein (i.e., added to
30 SEQ ID NO:2) or an ATG added to the 5' end of the nucleotide sequence (i.e., added to SEQ ID NO:1), in order to express the channel in a host cell. The met version of the mature channel is thus specifically

intended to be covered by reference to SEQ ID NO:1 or SEQ ID NO:2.

The invention further provides an antibody or fragment thereof specific for the pancreatic T-type calcium channel of the subject invention. Antibodies of the subject invention include polyclonal antibodies and monoclonal antibodies capable of binding to the pancreatic T-type calcium channel, as well as fragments of these antibodies, and humanized forms. Humanized forms of the antibodies of the subject invention may be generated using one of the procedures known in the art such as chimerization. Fragments of the antibodies of the present invention include, but are not limited to, the Fab, the F(ab')₂, and the Fc fragments.

The invention also provides hybridomas which are capable of producing the above-described antibodies. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (see Campbell 1984 and St. Groth et al. 1980). Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with the antigenic pancreatic T-type calcium channel (or an antigenic fragment thereof). Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the protein. One skilled in the art will recognize that the amount of the protein used for immunization will vary based on the animal which is immunized, the antigenicity of the protein, and the site of injection.

The protein which is used as an immunogen may be modified or administered in an adjuvant in order to

increase the protein's antigenicity. Methods of increasing the antigenicity of a protein are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as a globulin or beta-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/O-Ag 15 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz et al. 1988).

Hybridomas secreting the desired antibodies are cloned and the class and subclass are determined using procedures known in the art (Campbell 1984).

For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

The present invention further provides the above-described antibodies in detectably labeled form.

Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.), fluorescent labels (such as FITC or rhodamine, etc.), paramagnetic atoms, etc.

Procedures for accomplishing such labeling are well known in the art, for example see Sternberger et al. 1970, Bayer et al. 1979, Engval et al. 1972, and Goding 1976.

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The labeled antibodies or fragments thereof of the present invention can be used for in vitro, in vivo, and in situ assays to identify cells or tissues which express pancreatic T-type calcium channel, to identify samples
5 containing pancreatic T-type calcium channel, or to detect the presence of pancreatic T-type calcium channel in a sample. More particularly, the antibodies or fragments thereof can thus be used to detect the presence of pancreatic T-type calcium channel in a sample, by
10 contacting the sample with the antibody or fragment thereof. The antibody or fragment thereof binds to any pancreatic T-type calcium channel present in the sample, forming a complex therewith. The complex can then be detected, thereby detecting the presence of pancreatic T-
15 type calcium channel in the sample. As will be readily apparent to those skilled in the art, such a method could also be used quantitatively to assess the amount of pancreatic T-type calcium channel in a sample. As should also be readily apparent, such an antibody can also be
20 used to decrease levels of functional T type calcium channels, by blocking the channel. Such antibodies can therefore be used in the methods of the subject invention to modify levels of functional T type calcium channels in pancreatic beta cells.

25 Further provided is a composition comprising the pancreatic T-type calcium channel protein and a compatible carrier.

In the methods of the invention, tissues or cells are contacted with or exposed to the composition of the
30 subject invention or a compound. In the context of this invention, to "contact" tissues or cells with or to "expose" tissues or cells to a composition or compound means to add the composition or compound, usually in a liquid carrier, to a cell suspension or tissue sample,

either in vitro or ex vivo, or to administer the composition or compound to cells or tissues within an animal (including humans).

For therapeutics, methods of modifying insulin
5 secretion by pancreatic beta cells, methods of treating —
type II diabetes, methods of modifying basal calcium
levels in cells, methods of modifying the action
potential of L type calcium channels in cells, methods of
modifying pancreatic beta cell death, methods of
10 modifying pancreatic beta cell proliferation, and methods
of modifying calcium influx through L type calcium
channels in cells, each of the methods comprising
modifying levels of functional T type calcium channels in
the cells, are provided. The formulation of therapeutic
15 compositions and their subsequent administration is
believed to be within the skill in the art. In general,
for therapeutics, a patient suspected of needing such
therapy is given a composition in accordance with the
invention, commonly in a pharmaceutically acceptable
20 carrier, in amounts and for periods which will vary
depending upon the nature of the particular disease, its
severity and the patient's overall condition. The
pharmaceutical compositions of the present invention may
be administered in a number of ways depending upon
25 whether local or systemic treatment is desired and upon
the area to be treated. Administration may be topical
(including ophthalmic, vaginal, rectal, intranasal,
transdermal), oral or parenteral. Parenteral
administration includes intravenous drip or infusion,
30 subcutaneous, intraperitoneal or intramuscular injection,
pulmonary administration, e.g., by inhalation or
insufflation, or intrathecal or intraventricular
administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders.

Conventional pharmaceutical carriers, aqueous, powder or
5 oily bases, thickeners and the like may be necessary or —
desirable. Coated condoms, gloves and the like may also
be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-
10 aqueous media, capsules, sachets or tablets. Thickeners,
flavoring agents, diluents, emulsifiers, dispersing aids
or binders may be desirable.

Compositions for parenteral, intrathecal or
intraventricular administration may include sterile
15 aqueous solutions which may also contain buffers,
diluents and other suitable additives.

In addition to such pharmaceutical carriers,
cationic lipids may be included in the formulation to
facilitate uptake. One such composition shown to
20 facilitate uptake is LIPOFECTIN (BRL, Bethesda MD).

Dosing is dependent on severity and responsiveness
of the condition to be treated, with course of treatment
lasting from several days to several months or until a
cure is effected or a diminution of disease state is
25 achieved. Optimal dosing schedules can be calculated
from measurements of drug accumulation in the body.
Persons of ordinary skill can easily determine optimum
dosages, dosing methodologies and repetition rates.
Optimum dosages may vary depending on the relative
30 potency of individual compositions, and can generally be
calculated based on IC_{50} 's or EC_{50} 's in in vitro and in
vivo animal studies. For example, given the molecular
weight of compound (derived from oligonucleotide sequence
and/or chemical structure) and an effective dose such as

an IC_{50} , for example (derived experimentally), a dose in mg/kg is routinely calculated.

The methods of the subject invention are based on the discovery that regulation of T type calcium channels directly modifies basal calcium levels in cells, which in turn regulates L type calcium channel activity, which in turn regulates insulin secretion and cell death, which in turn treats type II diabetes. The methods of the subject invention are further based on the discovery that regulation of T type calcium channels directly affects basal and glucose-induced insulin secretion.

T type calcium channels belong to the family of low voltage activated calcium channels. Modifying (increasing or decreasing) "levels" of functional T type calcium channels refers to modifying expression of the T type calcium channel gene, modifying activity of the T type calcium channel such as by inhibiting the function of the channel, and/or modifying the formation of active membrane-spanning T type calcium channels. As used herein, "functional" refers to the synthesis and any necessary post-translational processing of a calcium channel molecule in a cell so that the channel is inserted properly in the cell membrane and is capable of conducting calcium ions in accordance with a low voltage activated channel.

The invention thus provides a method of modifying insulin secretion by pancreatic beta cells, the method comprising modifying levels of T type calcium channels in the pancreatic beta cells.

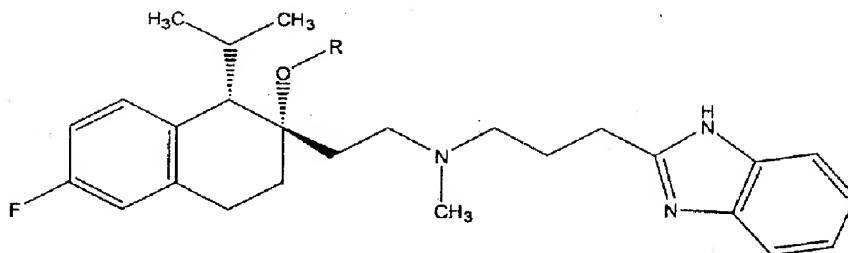
Levels of T type calcium channels in the pancreatic beta cells can be modified by various methods, at the gene and protein and "functional calcium channel" levels. In one embodiment, the levels are modified by modifying T type calcium channel gene expression of the T type

calcium channel in the cells. This can be accomplished by exposing the cells to a compound which modifies T type calcium channel gene expression of the calcium channel. The compound could be, for example, an antisense
5 oligonucleotide targeted to the T type calcium channel gene. In a similar embodiment, the compound which modifies T type calcium channel gene expression of the T type calcium channel could be a ribozyme.

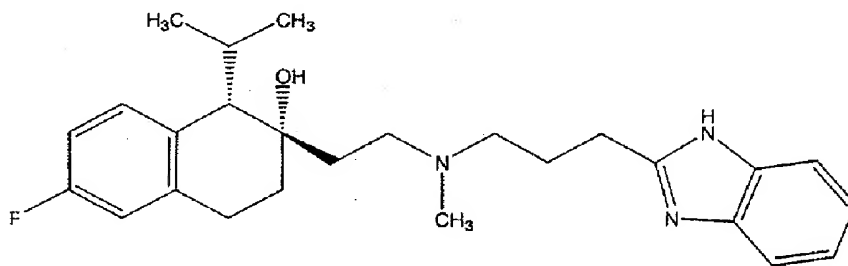
Other methods for modifying T type calcium channel
10 gene expression could also involve site-directed mutagenesis of the T type calcium channel gene to prevent expression of the T type calcium channel, or various gene therapy techniques.

Levels, in particular activity, of T type calcium
15 channels in the cell can also be modified by exposing the cells to an inhibitor of the T type calcium channel. Such inhibitors include, for example, mibefradil, mibefradil analogs, amiloride, NiCl_2 , and second messengers which regulate activity of the T type calcium
20 channels. Other inhibitors of the T type calcium channel could also readily be identified by screening methods (including the method described above). In addition to chemical inhibitors, peptide inhibitors could also be identified with screening methods (for example, using
25 phage display libraries and other peptide screening methods).

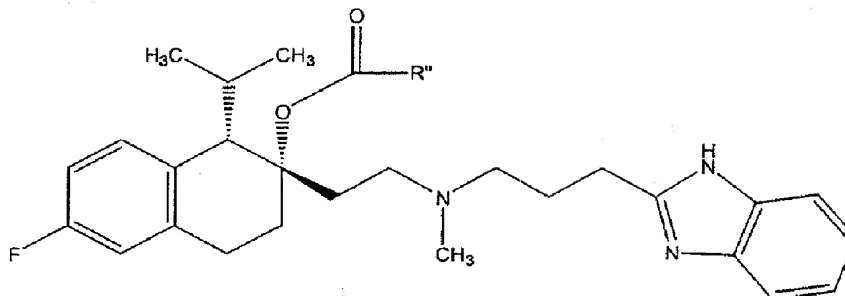
"Mibefradil analogs", as used herein are meant to include compounds having the formula:



wherein R is hydrogen, alkyl, or a moiety having the formula C(O)R', where R' is alkyl or aryl. In the above
 5 formulae, alkyl is meant to include linear alkyls, particularly C1-C12 linear alkyls (e.g., methyl, ethyl, n-propyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, n-nonyl, and the like), branched alkyls, particularly C1-C12
 branched alkyls (e.g., isobutyl, isopentyl, neopentyl,
 10 hex-2-yl, hex-3-yl, hept-2-yl, hept-3-yl, and the like), and cycloalkyls, particularly C1-C8 cycloalkyls (e.g., cyclopentyl, cyclohexyl, cycloheptyl, 4-methylcyclohexyl, and the like). These alkyl groups can be substituted or unsubstituted. When substituted, suitable substituents
 15 include, for example, aryl groups, halogen atoms, hydroxy groups, alkoxy groups, carboxylic acid groups, amine groups, and the like, as well as combinations of these substituents. Mibefradil analogs which are particularly well suited to blocking (inhibiting) the activity of T-
 20 type calcium channels but not blocking the activity of L-type calcium channels are those having the formula:



and those having the formula:



- 5 in which R'' is an unsubstituted alkyl group or a substituted alkyl group which does not contain an alkoxy substituent. "Mibefradil analogs" are also meant to include compounds having the above formulae which are substituted at other positions in the structure, for
- 10 example, on the benzimidazole phenyl moiety, at a benzimidazole nitrogen, at other positions of the tetrahydronaphthyl ring, etc. Also included within the meaning of "mibefradil analogs" are compounds having the above formulae in which the F is replaced with another
- 15 substituent, such as another halogen. Also included within the meaning of "mibefradil analogs" are compounds having the above formulae in which the amine methyl group or the isopropyl group or both are replaced with other substituents, such as other alkyl moieties.
- 20 Additionally, "mibefradil analogs" are meant to include those compounds which are generically described and/or specifically disclosed in U.S. Patent No. 4,808,605, which is hereby incorporated by reference. Further, "mibefradil analogs" are meant to include
- 25 pharmaceutically acceptable salts of the derivatives described above. Illustrative pharmaceutically acceptable salts are salts formed with hydrochloric acid, hydrobromic acid, nitric acid, sulphuric acid, phosphoric acid, citric acid, formic acid, maleic acid, acetic acid,

succinic acid, tartaric acid, methanesulphonic acid, p-toluenesulphonic, and the like.

Mibefradil analogs can be made by following the general procedures described in, for example, U.S. Patent
5 Nos. 4,808,605, 5,910,606, 5,892,055, 5,811,557, —
5,811,556, and 5,808,088, each of which is hereby incorporated by reference.

Levels of T type calcium channels in the cell can also be modified by exposing the cells to a compound
10 which interferes with membrane T type calcium channel formation.

Levels of functional T type calcium channel could also be modified by use of molecules which bind to transcription regulators of the T type calcium channel
15 gene (such as the promoter region of the gene).

The invention further provides a method of treating type II diabetes in a subject (human or animal), the method comprising administering to the subject an amount of a compound effective to modify levels of T type
20 calcium channels in the pancreatic beta cells of the subject. As above, the compound may modify levels of T type calcium channels by modifying T type calcium channel gene expression of the calcium channel, or by inhibiting the T type calcium channel, or by interfering with
25 membrane T type calcium channel formation.

In the context of this invention "modulation" or "modifying" means either inhibition or stimulation. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA
30 expression, Western blot assay of protein expression, or calcium channel activity assay.

The compounds and/or inhibitors used in the methods of the subject invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any

other compound/inhibitor which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, 5 the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds and/or inhibitors used in the subject invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

10 In regard to prodrugs, the compounds and/or inhibitors for use in the invention may additionally or alternatively be prepared to be delivered in a prodrug form. The term prodrug indicates a therapeutic agent that is prepared in an inactive form that is converted to 15 an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions.

In regard to pharmaceutically acceptable salts, the term pharmaceutically acceptable salts refers to 20 physiologically and pharmaceutically acceptable salts of the compounds and/or inhibitors used in the subject invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

25 Drugs, such as peptide drugs, which inhibit the T type calcium channel or which interfere with functional T type calcium channel formation can be identified by other methods also. For example, a monoclonal antibody can be prepared which specifically hybridizes to the T type 30 calcium channel, thereby interfering with activity and/or channel formation. Once a monoclonal antibody which specifically hybridizes to the T type calcium channel is identified, the monoclonal (which is itself a compound or inhibitor which can be used in the subject invention) can

be used to identify peptides capable of mimicking the inhibitory activity of the monoclonal antibody. One such method utilizes the development of epitope libraries and biopanning of bacteriophage libraries. Briefly, attempts
5 to define the binding sites for various monoclonal antibodies have led to the development of epitope libraries. Parmley and Smith developed a bacteriophage expression vector that could display foreign epitopes on its surface (Parmley, S.F. & Smith, G.P., Gene 73:305-318
10 (1988)). This vector could be used to construct large collections of bacteriophage which could include virtually all possible sequences of a short (e.g. six-amino-acid) peptide. They also developed biopanning, which is a method for affinity-purifying phage displaying
15 foreign epitopes using a specific antibody (see Parmley, S.F. & Smith, G.P., Gene 73:305-318 (1988); Cwirla, S.E., et al., Proc Natl Acad Sci USA 87:6378-6382 (1990); Scott, J.K. & Smith, G.P., Science 249:386-390 (1990); Christian, R.B., et al., J Mol Biol 227:711-718 (1992);
20 Smith, G.P. & Scott, J.K., Methods in Enzymology 217:228-257 (1993)).

After the development of epitope libraries, Smith et al. then suggested that it should be possible to use the bacteriophage expression vector and biopanning technique
25 of Parmley and Smith to identify epitopes from all possible sequences of a given length. This led to the idea of identifying peptide ligands for antibodies by biopanning epitope libraries, which could then be used in vaccine design, epitope mapping, the identification of
30 genes, and many other applications (Parmley, S.F. & Smith, G.P., Gene 73:305-318 (1988); Scott, J.K., Trends in Biochem Sci 17:241-245 (1992)).

Using epitope libraries and biopanning, researchers searching for epitope sequences found instead peptide

sequences which mimicked the epitope, i.e., sequences which did not identify a continuous linear native sequence or necessarily occur at all within a natural protein sequence. These mimicking peptides are called
5 mimotopes. In this manner, mimotopes of various binding sites/proteins have been found.

The sequences of these mimotopes, by definition, do not identify a continuous linear native sequence or necessarily occur in any way in a naturally-occurring
10 molecule, i.e. a naturally occurring protein. The sequences of the mimotopes merely form a peptide which functionally mimics a binding site on a naturally-occurring protein.

Many of these mimotopes are short peptides. The
15 availability of short peptides which can be readily synthesized in large amounts and which can mimic naturally-occurring sequences (i.e. binding sites) offers great potential application.

Using this technique, mimotopes to a monoclonal
20 antibody that recognizes T type calcium channels can be identified. The sequences of these mimotopes represent short peptides which can then be used in various ways, for example as peptide drugs that bind to T type calcium channels and decrease the activity of T type calcium
25 channels. Once the sequence of the mimotope is determined, the peptide drugs can be chemically synthesized.

MATERIALS AND METHODS

30 Cell Culture - INS-1 cells were cultured in RPMI 1640 medium containing 10% FBS, 25 U/ml penicillin, 25 mg/ml streptomycin and 50 μ M mercaptoethanol in an atmosphere of 5% CO₂ in air, at 37°C for 2-5 days before recording.

Islet cell preparation - Pancreases of Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) were removed after intrapancreatic perfusion with 2 ml of Hanks' solution (Gibco BRL, Grand Island, NY) containing collagenase (4 mg/ml, Boehringer - Mannheim, Indianapolis, IN), DNase I (10 µg/ml, Sigma, St. Louis, MO), CaCl₂ (1.28 mM) and bovine serum albumin (1 mg/ml, Gibco BRL). The pancreatic tissue was incubated at 37°C for 20 min and then washed five times with enzyme-free Hanks' solution. Islets were picked up and treated with 0.1% pancreatin (Sigma) for five minutes at 37°C. Single cells were obtained by triturating the islets with plastic pipette tips and then they were transferred into 35 mm culture dishes. Cells were cultured in RPMI 1640 medium (Gibco BRL) containing 5 mM glucose, 10% FBS and P/S at 37°C, 5% CO₂ for 2-5 days before experiments.

Isolation of RNA - Total RNA was isolated from cultured INS-1 cells and from various freshly excised rat tissues by the guanidinium isothiocyanate/phenol procedure (Chomczynski and Sacchi 1987). Poly-A RNA was isolated from total RNA by two successive passes over an oligo (dT)-cellulose spin column (Ambion, Austin, TX).

Cloning of cDNA Encoding α_1 Subunit of T-type Ca²⁺ channel in INS-1 - First strand cDNA was prepared using 2 µg of INS-1 cell mRNA and M-MLV reverse transcriptase (Gibco BRL) with the poly-dT primers. The first 433 bp DNA fragment of the channel was deduced with PCR using the degenerate primers (forward) (SEQ ID NO:6) 5'-TNGC(A/C/T)ATGGAG(C/A)GNCC(C/T)-3' and (backward) (SEQ ID NO:7) 5'-CTT(C/G/T)CCCTTGAA(G/C)A(G/A)CTG)-3' based on conserved voltage-dependent Ca²⁺ channel α_1 subunit sequences in domain III. Using the Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA), the 3'- and

5'- rapid amplifications of cDNA end-PCR (RACE-PCR) were performed to obtain the entire gene of the α_1 subunit of the channel. For the 5'-RACE-PCR, the forward primer was an adapter primer, the backward primer was (SEQ ID NO:8) 5'-CCGCTGTCTCGGAGACCATGGAGACC-3'; for the 3'-RACE, the forward primer was (SEQ ID NO:9) 5'-AGCGGCCCAAATTGACCCCCACAG-3' and the backward primer was poly-dT. The RT-PCR products were subcloned into pT-Adv Vector (Clontech) and dideoxynucleotide sequencing assay was performed with a dsDNA Cycle Sequencing System (Gibco BRL).

Tissue distribution - The gene expression of T-type Ca^{2+} channels deduced from β -cells was examined in rat brain, heart, kidney, and liver using an RT-PCR assay. The primers used for the RT-PCR were (SEQ ID NO:10) 5'-GAAGATGCGAGTGGACAG-3' (forward) and (SEQ ID NO:11) 5'-CTGTGGCGATGGTCACTG-3' (backward). The PCR products were detected by agarose gel electrophoresis on a 1% gel.

Genome walking - The genome walker library (Clontech) was used as a template in nested PCR reactions with gene-specific primers (GSP) and the adapter primers (AP) provided with the kit. The first PCR reaction was carried out in 5 tubes, each having a total volume of 50 μl : 5 μl 10X PCR reaction buffer, 1 μl dNTP (10 mM each), 2.2 μl $\text{Mg}(\text{OAc})_2$ (25 mM), 1 μl AP1 (10 μM), 1 μl GSP1, 1 μl Advantage Genomic Polymerase Mix (50X), and 37.8 μl water. The following two-step cycle parameters were used: (Step 1) 7 cycles of denaturing at 94°C for 25 sec., annealing and extension at 72°C for 4 min. (Step 2) 32 cycles of denaturing at 94°C for 25 sec., annealing and extension at 67°C for 4 min. After the second step cycle, the samples were held at 67°C for 4 min. The second PCR reaction was carried out under the reaction condition similar to the first PCR reaction except using

AP2, GSP2. In addition, the templates used were 1 μ l of 1:50 dilution of each primary PCR reaction. The two step cycles were similar to the first PCR reaction except 5 cycles at the first step and 22 cycles at the second 5 step.

Oocyte electrophysiology - cRNA transcripts were synthesized from BssH II linearized pT-Adv cDNA templates using T7 RNA polymerase (Ambion). Defolliculated *Xenopus laevis* were injected with 25 ng pT-Adv cRNA. Three to 10 five days after injection, two-electrode voltage-clamp recording was performed using a Warner OC-725C amplifier (Warner Instrument Corp., Hamden, CT). Data were acquired and analyzed with Pulse/PulseFit software (HEKA, Lambrecht/Pfalz, Germany). The bath solution contained 15 the following: 40 mM $\text{Ca}(\text{OH})_2$, 50 mM NaOH, 2 mM TEA-Cl, 1mM KOH, 0.1 mM EDTA and 5 mM HEPES, adjusted to pH 7.4 with methanesulphonate. Boltzmann fits were calculated using Prism (GraphPad). Results are presented as mean \pm s.d. unless otherwise stated.

20 β -cell Electrophysiological recording - The whole-cell recordings were carried out by the standard "giga-seal" patch clamp technique (Hamill et al.). The whole-cell recording pipettes were made of hemocapillaries (Warner), pulled by a two-stage puller 25 (PC-10, Narishige International, New York, NY), and heat polished with a microforge (MF200-1, World Precision Instruments, Sarasota, FL) before use. Pipette resistance was in the range of 2-5 M Ω in the internal solution. The recordings were performed at room temperature (22-25°C). 30 Currents were recorded using an EPC-9 patch-clamp amplifier (HEKA) and filtered at 2.9 kHz. Data were acquired with Pulse/PulseFit software (HEKA). Voltage-dependent currents were corrected for linear leak

and residual capacitance by using an on-line P/n subtraction paradigm.

Drugs - Mibefradil ((1S,2S)-2-[2-[[3-(2-Benzimidazolyl)propyl]methyl-amino]ethyl]-6-fluoro-
5 1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxy-acetate -
dihydrochloride) was kindly provided by Dr. J.-P. Clozel
(Hoffmann LaRoche, Basel, Switzerland), and can be
synthesized according to the methods disclosed in U.S.
Patent Nos. 5,892,055, 5,811,557, 5,811,556, and
10 5,808,088. U.S. Patent No. 4,808,605 describes
mibefradil compounds suitable for use in the subject
invention.

The free alcohol Des-methoxyacetyl mibefradil
(1S,2S)-2-[2-[[3-(2-Benzimidazolyl)propyl]
15 methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-
isopropyl-2-naphthyl hydroxy hydrochloride) was prepared
by alkaline hydrolysis: 14.2 mg mibefradil hydrochloride
was dissolved in 4 ml methanol + 1 ml 10 N aqueous sodium
hydroxide mixture (5 mM was the final concentration of
20 mibefradil). The solution was warmed in a boiling water
bath for 10 min. The reaction was followed by mass
spectrometry. Upon completion of the hydrolysis, as
determined from the mass spectra, the solution was
neutralized with 5 M aqueous hydrochloric acid. The
25 slight loss of methanol that occurred by evaporation
during the reaction was corrected by adding water to keep
the total volume of 5 ml.

Solutions - The extracellular solution used in
whole-cell Ca^{2+} current recording contained (in mM): 10
30 CaCl_2 , 110 tetraethylammonium-Cl (TEA-Cl), 10 CsCl, 10 N-
2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
(HEPES), 40 sucrose, 0.5 3,4-diaminopyridine, pH 7.3. The
intracellular solution contained (in mM): 130 N-methyl-D-
glucamine, 20 EGTA (free acid), 5 bis (2-aminophenoxy)

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ethane-N, N, N', N'-tetraacetate (BAPTA), 10 HEPES, 6 MgCl₂, 4 Ca(OH)₂, pH was adjusted to 7.4 with methanesulfonate. 2 mM Mg-ATP was included in the pipette solution to minimize rundown of L-type Ca²⁺ currents. For
5 Perforated-patch recording, the extracellular solution — contained (in mM): 26 Sucrose, 30 TEA-Cl, 10 HEPES, 5 KCl, 2 CaCl₂, MgCl₂, pH 7.3. The pipette solution contained (in mM): 65 CsOH, 65 CsMS, 20 sucrose, 10 HEPES, 10 MgCl₂, 1 Ca(OH)₂, pH 7.4.

10 Mass Spectrometric Analysis - A VG 70-250 SEQ instrument (VG Analytical, Manchester, UK) was used with fast atom bombardment (FAB) ionization mode to obtain mass spectra of the mibefradil and dm-mibefradil. Cultured INS-1 cells were treated with 20 μM mibefradil
15 for various lengths of time under each experimental condition. The cell pellets were collected after washing three times with PBS and resuspended in 0.5 ml media for mass spectrometric analysis. For a 50 μl cell sample, 20 μl internal standard solution (40 μM verapamil, MW:454)
20 and 5 μl glycerol was added, and 4 μl of this mixture was used for FAB-MS. Several positive ion spectra were recorded in the mass range m/z 750-100 at a mass resolution of 1000, and a scan speed of 2 second/decade. For mibefradil, m/z 496 was the dominant ion (M+H)⁺
25 accompanied with a less intense sodiated molecular ion m/z 518. The concentrations of the mibefradil and hydrolyzed mibefradil were obtained by comparing the intensities of m/z 496 and 424 were to the intensity of m/z 455. For calibration, a standard solution of 50 μM
30 drug was subjected to mass spectrometric analysis.

Separation of cytosolic and membrane components - After washing out mibefradil from the bath solution, the cells were collected and the membranes were broken down by vortexing the cells in a solution containing 5% acetic

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acid/ CH_3CN . The mixture was then spun and the supernatant collected and defined as non-membrane associated components. Pellets were re-suspended in 5 x volume of NaOH (10 N):methanol (1:7) solution at 37°C for 5 min.

- 5 The mixture was neutralized with 0.5 M HCl and spun down.—
The remaining pellet and the supernatant were collected separately.

Statistics - All data is presented as mean \pm s.d. and the student's t-test was used to calculate p values
10 where given.

EXAMPLE I

Identification and Cloning of a Pancreatic T-type Calcium Channel

- 15 The subject invention provides a cDNA encoding a T-type Ca^{2+} channel α_1 subunit derived from the rat insulin secreting cell line, INS-1, which has been identified and sequenced. The sequence of the cDNA indicates a protein composed of 2288 amino acids (SEQ ID
20 NO:2), sharing 96.3% identity to the neuronal T-type Ca^{2+} channel α_1 subunit ($\alpha_1\text{G}$). The transmembrane domains of the protein are highly conserved but the isoform contains three distinct regions as well as 10 single amino acid substitutions in other regions. Sequencing rat genomic
25 DNA revealed that this is an alternative splice isoform of $\alpha_1\text{G}$. Using specific primers and reverse transcription polymerase chain reaction (RT-PCR) it was demonstrated that both splice variants are expressed in rat islets. The isoform deduced from INS-1 was also expressed in
30 brain, neonatal heart and kidney. Functional expression of this $\alpha_1\text{G}$ isoform in *Xenopus* oocytes generated low-voltage activated Ca^{2+} currents. These results provide the molecular biological basis for studies of function of

T-type Ca^{2+} channels in β -cells where these channels play critical roles in diabetes.

The cloning and tissue distribution of an isoform of the T-type Ca^{2+} channel ($\alpha_1\text{G-INS}$) derived from the rat
5 insulin-secreting cell line, INS-1 (Asfari et al. 1992), — is described further below.

Based on the conserved amino acid sequence comprising the six transmembrane segments in repeat III of the previously cloned α_1 -subunit (Stea et al. 1995),
10 degenerate primers were designed to deduce the cDNA sequence of voltage-dependent Ca^{2+} channel from INS-1 which expresses a high level of T-type Ca^{2+} current (Bhattacharjee et al. 1997). A 433 base pair (bp) DNA fragment was obtained. The rapid amplification of cDNA
15 ends (RACE) strategy was then used to obtain the entire sequence of the channel. The full length cDNA (SEQ ID NO:1) encodes a protein containing 2288 amino acids (SEQ ID NO:2).

The T-type Ca^{2+} channel gene deduced from β -cells
20 shares 96.3% amino acid identity with $\alpha_1\text{G}$, the neuronal isoform of T-type Ca^{2+} channel (Perez-Reyes et al. 1998). The four intramolecular homologous transmembrane domains of β -cell T-type Ca^{2+} channel α_1 subunit are identical (except glycine 1667) to $\alpha_1\text{G}$, with each repeat containing
25 six putative membrane-spanning regions (S1-S6) and a pore-forming region (P-loop). The other highly conserved region is located at the intracellular loop between repeat I and II, where a section of histidine-rich chain is present in the β -cell derived T-type Ca^{2+} channel gene
30 as well as in neuronal and cardiac T-type Ca^{2+} channel genes. This structure in the loop_{I-II} has not been observed in the protein sequences of known high voltage activated Ca^{2+} channels.

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In addition to the single amino acids that differ from α_1G , the T-type Ca^{2+} channel gene derived from β -cells contains three unique regions that differ from the amino acid sequence of α_1G . These regions are located
5 at the N-terminal amino acids (aa1-34 of SEQ ID NO:2),
intracellular loop I_{II-III} (aa971-994 of SEQ ID NO:2) and
intracellular loop L_{III-IV} (aa1570-1588 of SEQ ID NO:2).

Although the amino acid sequence of the deduced channel is entirely different from the α_1G in the
10 N-terminal region (aa1-34 of SEQ ID NO:2), the nucleotide
sequences at this region are almost identical except for
4 single nucleotide insertions which are shown in Fig.
1A. These four single nucleotide insertions determine a
different start codon as well as those of the amino acid
15 sequences.

To resolve the relationship between the T-type Ca^{2+} channel isoform deduced from INS-1 and α_1G , a section of Sprague-Dawley rat genomic DNA sequence containing the introns and exons between 4845 and 5256 was identified.
20 As shown in Fig. 1B, an exon was found that encodes the
 α_1G fragment SKEKQMA (SEQ ID NO:5) as well as an exon that
encodes fragment 4869-4922 of the INS-1 variant. This
region also contains 8.5 kilobases (kb) of intron
sequence. Thus, the T-type Ca^{2+} channel α_1 subunit cloned
25 from INS-1 and α_1G are alternative splice isoforms of the
same gene.

The genomic DNA sequence was also used to examine the two nucleotide discrepancy between the α_1G cDNA and the isoform cloned from INS-1. The data show that the
30 genomic nucleotide sequence encoding amino acid 1667 is
GGC (glycine), which is the same as the cDNA of α_1 subunit
cloned from INS-1 and the corresponding residue in α_1H ,
but is different from α_1G (GCG, alanine). Also of note,
there are nine additional single amino acid substitutions

in the isoform deduced from INS-1 as compared to the α_1G . Six correspond to the amino acids found in the analogous position of α_1H : cysteine 1088, glycine 1667, alanine 1700, aspartic acid 1735, threonine 1812, and leucine 5 1813.

In regard to tissue distribution of T-type Ca^{2+} channels deduced from β -cells and from neurons, expression of the β -cell T-type Ca^{2+} channel was found in rat brain, heart and kidney, but was absent from liver. 10 Both α_1G and the splice form were detected in rat islets and INS-1 cell preparations using RT-PCR. No α_1H was detected.

Functional expression of the T-type Ca^{2+} channels deduced from β -cells has been conducted in *Xenopus* 15 oocytes using a double-electrode voltage-clamp method. In a solution containing 40 mM Ca^{2+} , a family of current traces representing T-type Ca^{2+} current characteristics were obtained (Fig. 2A). The current slowly activated at -40 mV and peaked at -10 mV. The analysis of time 20 constants of activation and inactivation are shown in Fig. 2B. The voltage-dependent activation (Fig. 2C) and steady-state inactivation (Fig. 2D) were fitted with Boltzmann equation. The calculated $V_{1/2}$'s were -23.8 mV and -45.6 mV for activation and inactivation, 25 respectively; and k 's were 5.3 and -6.0 for activation and inactivation, respectively.

The nucleotide cDNA (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of rat pancreatic T-type calcium channel were determined. SEQ ID NO:3 is the nucleotide 30 sequence beyond the coding region, while SEQ ID NO:4 includes SEQ ID NO:2.

EXAMPLE II

Characterization of the T type Calcium Channel
in Relation to Diabetes

Glucose stimulated insulin release is Ca^{2+} dependent
5 process, involving closure of the ATP-sensitive potassium-
channels, depolarization and opening of the voltage-
dependent Ca^{2+} channels. At glucose concentrations below
3 mM, which do not elicit insulin secretion, β -cells are
electrically silent with a resting membrane potential of
10 about -70 mV. Raising external glucose produces a slow
depolarization, the extend dependent upon the glucose
concentration. At glucose levels which elicit insulin
release (>7 mM) depolarization is sufficient to reach the
threshold potential (-50 mV) at which electrical activity
15 is initiated.

A simple model for glucose-stimulated insulin
secretion is summarized in Fig. 12. The resting membrane
potential of β -cells is principally determined by the
activity of the K-ATP channel. When plasma glucose
20 rises, its uptake and rate of metabolism by β -cells are
stimulated. As a consequence, the intracellular ATP (or
ATP:ADP ratio) increases which leads to the closure of K-
ATP channels and membrane depolarization. This results
in the activation of voltage dependent Ca^{2+} channels (T-
25 type and L-type) and the initiation of electrical
activity. The increased calcium influx leads to a rise
in $[\text{Ca}^{2+}]_i$ and consequently insulin secretion.

Rat and human pancreatic β -cells are equipped with
L-type and T-type Ca^{2+} channels. The physiological
30 function of T-type Ca^{2+} channels in β -cells insulin-
secretion has been demonstrated. These channels
facilitate exocytosis by enhancing electrical activity in
these cells. L-type and T-type Ca^{2+} channels, under
normal conditions, work in concert promoting the rise in

[Ca²⁺]_i during glucose-stimulated insulin secretion. In β-cells, over-expressed T-type Ca²⁺ channels are, at least in part, responsible for the hyper-responsiveness of insulin secretion to non-glucose depolarizing stimuli in
5 GK rat, and in rat with NIDDM induced by neonatal injection of streptozotocin. However, over-expressed T-type calcium channels over time will ultimately lead to an elevation of basal Ca²⁺ through its window current properties. Therefore, there is a dual effect of T-type
10 Ca²⁺ channels in β-cells depending upon channel number and membrane potential.

Pharmacologically antagonizing T-type calcium channels is an appropriate treatment protocol for alleviating both insulin resistance and enhancement of
15 insulin secretion in NIDDM patients.

NIDDM pathogenesis is complex and the disease progression occurs in phases. An enhanced β-cell responsiveness provokes and initiates the disease process. It is unclear as to what the actual enhanced
20 activity is and what the triggering mechanisms are for this first phase. It may be an increased secretory response or an increase in β-cell mass. However, there is clearly an enhancement of β-cell activity detected by both basal and postprandial elevated insulin levels
25 denoted as hyperinsulinemia. Consequently, a resulting insulin resistance occurs, phase II, particularly in insulin responsive tissues (muscle, liver, kidney, fat) that function to reduce glucose levels in the blood. A decrease in insulin sensitivity will account for an
30 increase in blood glucose, causing the β-cells to secrete even more insulin to compensate and because of this vicious cycle, full blown NIDDM, marked by an inevitable defect in insulin release, hyperglycemia and insulin

resistance, will characterize the final stage of the disease process.

Each phase of the disease may be characterized by an alteration in $[Ca^{2+}]_i$, and each phase can be treated by a T-type calcium channel antagonist. The electrical β -cell—
5 is equipped with two types of voltage-dependent calcium channels, L-type and T-type calcium channels. L-type calcium channels, activated at high voltages, having large unitary conductance, and dihydropyridine-sensitive,
10 are considered the major pipeline for calcium influx into the β -cell (especially at high voltage depolarization). T-type calcium channels, activated at low voltages, with small unitary conductance, and dihydropyridine-insensitive, are important for maintaining basal $[Ca^{2+}]_i$,
15 (Fig. 8), as well as enhancing electrical activity during cell depolarization. T-type calcium channels normally facilitate insulin secretion in β -cells by enhancing cell electrical activity. This modulatory function of T-type calcium channels in insulin secretion is significant
20 during phase I prior to onset of diabetes. Antagonizing these T-type calcium channels will decrease β -cell hyper-responsiveness and consequent hyperinsulinemia arresting the pathogenic pathways that lead to NIDDM.

If hyperinsulinemia and associated insulin
25 resistance has already occurred, a T-type calcium channel blocker is still the appropriate treatment protocol. The insulin responsive tissues, those that are primarily responsible for taking up glucose for re-establishing euglycemia, have elevated basal $[Ca^{2+}]_i$ during
30 hyperinsulinemic conditions. Indeed, it is the elevated basal $[Ca^{2+}]_i$ that precipitates the decrease in insulin sensitivity of these tissues and it is now known that most of these insulin responsive tissues express T-type calcium channels. A T-type calcium channel blocker will

reduce the basal $[Ca^{2+}]_i$ and alleviate the decreased insulin sensitivity.

Once NIDDM has manifested, it is characterized by altered glucose metabolism, a result of abnormal glucose stimulus-secretion responsiveness of β -cells. β -cell desensitization to glucose is the principal secretory defect of NIDDM. L-type and T-type calcium channels, under normal conditions, work in concert promoting the rise in $[Ca^{2+}]_i$ during glucose-stimulated insulin secretion. In NIDDM, this partnership is broken and the necessary rise in $[Ca^{2+}]_i$ for insulin secretion is compromised.

The data herein indicates that L-type calcium channels are finely regulated by basal calcium levels (Figs. 9A-9D). A very small rise in basal calcium will substantially decrease the L-type calcium current and severely reduce the depolarization-induced rise in $[Ca^{2+}]_i$ (Figs. 10 and 11). The data herein also suggests that T-type calcium channels are a primary regulator of resting basal $[Ca^{2+}]_i$ in β -cells. Furthermore, the negative feedback regulation of T-type calcium channels by elevated $[Ca^{2+}]_i$ is absent (Figs. 9A-9D). It is under circumstances of enhanced T-type calcium current activity as seen in the GK rat model of NIDDM and in the neonate streptozotocin-induced diabetes model, that basal $[Ca^{2+}]_i$ is elevated, and a defect in the glucose-stimulated insulin secretion is observed. Simply reducing the basal calcium influx by pharmacological intervention, in situations of enhanced T-type calcium channel expression, may reduce basal $[Ca^{2+}]_i$ in β -cells (Fig. 8) and alleviate the $[Ca^{2+}]_i$ -induced inhibition of L-type calcium channels.

There is a clear link between $[Ca^{2+}]_i$ and diabetes. A primary abnormality in $[Ca^{2+}]_i$ handling by cells is the defect initiating parallel impairments in insulin

secretion and insulin action, as well as initiating diabetic complications. Consequent metabolic derangements may further aggravate alterations in $[Ca^{2+}]_i$ homeostasis, creating a relentless cycle leading to progressive deterioration in the overall health of the diabetic patient. Pharmacological agents that regulate $[Ca^{2+}]_i$ homeostasis are thus appropriate therapeutic measures. The use of T-type calcium channel blockers will thus effectively treat and perhaps cure diabetes mellitus.

EXAMPLE III

Pharmacology of Mibefradil Action

It has been shown that mibefradil has a potent inhibitory effect on T-type Ca^{2+} current in vascular smooth muscle cells. The data herein demonstrates that, in convention whole cell patch clamp configuration, mibefradil also blocks T-type Ca^{2+} current in pancreatic β -cells. Mibefradil ($1 \mu M$) had been administered in the recording chamber at time zero (Fig. 13), the control (no drug) showed "run down". This figure shows that T-type Ca^{2+} current is more sensitive to mibefradil than the L-type Ca^{2+} current in pancreatic β -cells.

The blockade of T-type Ca^{2+} channels in β -cells with mibefradil is reversible. Fig. 14 demonstrates the reversibility of blockade of T-type Ca^{2+} currents by mibefradil. In these experiments, a very little volume of mibefradil or $NiCl_2$ was delivered near the recording cell. The drug then diffused away from the cell. The final concentration in the chamber was 1 nM. This experiment shows the inhibitory effect of mibefradil on T-type Ca^{2+} current in pancreatic β -cells results from reversible interaction between the drug and the channel protein.

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In β -cells, T-type Ca^{2+} channels could mediate a small, but sustained, Ca^{2+} influx by means of their unique "window" current at voltages near resting membrane potentials. Like other voltage-regulated channels, T-type Ca^{2+} channels are opened and closed depending upon the potentials across the cell membranes. This voltage dependency is illustrated in Fig. 15. The activation and inactivation curves represent the percentage of the channels in either open or closed states over a range of voltages. Unlike most of the voltage-dependent Na^{+} channels or L-type Ca^{2+} channels, the activation and inactivation curves of T-type Ca^{2+} channels overlap at the certain range of low voltages (i.e. window). In other words, there is a small portion of T-type Ca^{2+} channels that stay in non-inactivated states in this voltage range. The data in Fig. 15 was obtained from experiments conducted under 10 mM external Ca^{2+} condition, which shifted the window current about 10 mV toward positive voltage due to the surface charge effects of divalent ions on the channels.

The existence of a window current provides a negative feedback regulation of $[\text{Ca}^{2+}]_i$ in β -cells. When cells are under an unhealthy condition, they may be slightly depolarized to activate window current, which elevates the basal $[\text{Ca}^{2+}]_i$ to protect the cells from further Ca^{2+} influx through the L-type Ca^{2+} channels. This process is reversible if the membrane potential is reset to the normal resting potential (-70 mV).

Mibefradil regulates basal $[\text{Ca}^{2+}]_i$ in pancreatic β -cells:

The data herein demonstrates the roles of T-type calcium currents in modulating basal $[\text{Ca}^{2+}]_i$ in INS-1 cells (Fig. 8). $[\text{Ca}^{2+}]_i$ was directly measured by the ratio of fluorescence excitations at Ca^{2+} -bound (380 nm)

to unbound (340 nm), and then the ratio was converted to the calcium concentration. The bath solution contained 10 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , and 2 mM MgCl_2 . In a single cell exhibiting fluctuating basal $[\text{Ca}^{2+}]_i$ with an average value near 150 nM, administering 1 μM mibefradil — into the chamber immediately lowered the basal calcium. This data shows the T-type calcium currents participate in regulating the mean basal $[\text{Ca}^{2+}]_i$ in cultured β -cells.

10 Mibefradil regulates basal insulin secretion:

The activation of T-type Ca^{2+} channel at low voltage near the resting membrane potential of pancreatic β -cells suggests that the channels are responsible for the Ca^{2+} influx required for insulin secretion under non-stimulus conditions. The NIT-1 cell line was chosen to demonstrate the effect of mibefradil on the basal insulin secretion. NIT-1 is a cell line derived from the β -cell of non-obese-diabetic mouse. This cell line expressed high levels of T-type Ca^{2+} current. The data herein shows that 5 μM mibefradil reduced the basal insulin secretion to less than 40% of control (Fig. 17), indicating this drug is able to lower the high basal insulin secretion level seen during the earlier stage of NIDDM.

25 Spontaneous elevation of $[\text{Ca}^{2+}]_i$:

To demonstrate that T-type Ca^{2+} channels play an important role in calcium entry under non-stimulatory conditions, and therefore regulate basal $[\text{Ca}^{2+}]_i$, spontaneous elevations of intracellular free calcium concentration was detected with the Fluo-3 AM fluorescent imaging method. NIT-1 cells were cultured in medium containing 3.3 mM glucose and preloaded with 2.5 μM Fluo-3 AM. The numbers of spontaneous calcium elevated cells were counted and compared to the total cells being used

for a 10 minute observation period. 10 μ M NiCl_2 inhibited 90% of spontaneous elevation of basal Ca^{2+} .

The cellular mechanism of the spontaneous elevation of intracellular Ca^{2+} was investigated with the epi-
5 fluorescence measurement method. Some INS-1 cells were -- observed to exhibit transient spontaneous elevations of $[\text{Ca}^{2+}]_i$, "Calcium spikes", under non-stimulatory conditions. The Role of T-type Ca^{2+} channels in this spontaneous process was examined as well. In a single
10 cell with spontaneous calcium spike activity (Fig. 17), NiCl_2 (30 μ M) reduced the frequency of spontaneous calcium spikes immediately. This result suggests that either the T-type Ca^{2+} channels alone or together with the L-type Ca^{2+} channels are responsible for the transient spontaneous
15 elevation of $[\text{Ca}^{2+}]_i$, under conditions where no glucose is present. These spontaneous calcium spikes may contribute to basal insulin secretion and control of basal $[\text{Ca}^{2+}]_i$.

However, neither mibefradil nor NiCl_2 exhibited their effect on basal $[\text{Ca}^{2+}]_i$ in all of the β -cells. It was
20 observed that only those cells which had relatively higher initial basal $[\text{Ca}^{2+}]_i$ will respond to the T-type Ca^{2+} channel antagonists (Fig. 18). Whereas those cells with lower initial basal $[\text{Ca}^{2+}]_i$ had no or less response to the T type Ca^{2+} channel antagonists. This result
25 indicates that T type Ca^{2+} channel antagonists may selectively act on the cells with high basal $[\text{Ca}^{2+}]_i$ and bring it back to normal, by inhibiting the window current.

30

EXAMPLE IV

Action on Pancreatic β -cells

T type Ca^{2+} may play two pathological roles in NIDDM. At the earlier stage, the NIDDM patients exhibit

hyperinsulinemia and β -cell hyperexcitability. This may, at least in part, be due to increased activity of T type Ca^{2+} channel in β -cells. At the more developed NIDDM stage, over-expressed T type Ca^{2+} channel and membrane depolarization resulted from reduced generation of ATP, and may set up a window current in β -cells that causes chronic elevation of basal Ca^{2+} in the β -cells. The elevated basal Ca^{2+} will reduce the L-type Ca^{2+} activity and glucose induced insulin secretion.

10 It has been shown that mibefradil prevented and reversed development of hyperinsulinemia in rat. This result indicates this drug is a valuable candidate for the treatment of earlier stage NIDDM or for preventing NIDDM in the potential patients.

15 A series of experiments were conducted with INS-1 cells to show that T type Ca^{2+} facilitated insulin secretion by enhancing the general excitability of pancreatic β -cells. Particularly, activation of T type Ca^{2+} channels will increase the firing frequency of the depolarizing spikes mediated by opening L type Ca^{2+} channels (Fig. 19A). Activation of T type Ca^{2+} channel will also decrease the time of developing action potential elicited by up-threshold depolarizations (Fig. 19B).

25 To further establish that T type Ca^{2+} current enhances β -cell excitability, 100 μM NiCl_2 was administered to effectively block T type Ca^{2+} channels. In contrast to control experiments, NiCl_2 caused a delay in the onset of an action potential and a decrease in number of action potentials.

To directly demonstrate the role of T type Ca^{2+} current in glucose-induced insulin secretion, INS-1 cells were incubated with 11.1 mM glucose and variable concentrations of NiCl_2 , and insulin release was measured.

NiCl_2 reduced insulin secretion in a dose-dependent manner (Fig. 20A). On the other hand, clonal insulin secreting cells (HIT-T15, which did not consistently exhibit T type Ca^{2+} current) were not affected by NiCl_2 (Fig. 20B).

- 5 These results show that T type Ca^{2+} channels play an — important role in β -cell excitability and antagonists of T type Ca^{2+} channels (such as NiCl_2) will effectively reduce the excitability of β -cells.

Although T type Ca^{2+} channels facilitate insulin
10 secretion by enhancing general excitability of β -cells, the function of T type Ca^{2+} channels is a doubled-edged sword. Under the condition of over-expressed T type Ca^{2+} channel in β -cells, the function of the window current will become dominant and result in an elevation of basal
15 Ca^{2+} . High $[\text{Ca}^{2+}]_i$ may cause impairment of insulin release by inactivating L type Ca^{2+} channels.

L-type Ca^{2+} channels are partially inactivated by $[\text{Ca}^{2+}]_i$ in non-stimulus condition in β -cells:

- 20 Upon establishment of a whole-cell patch, within the first five minutes, the L type Ca^{2+} current "runs-up", as the magnitude of the peak current increases over time in INS-1 cells (Fig. 21). This phenomenon is a universal feature in these cells under the recording conditions
25 used. The pipette solutions contained no ATP but did contain high concentrations of the calcium chelating agents BAPTA and EGTA. When the pipette solution contained high Ca^{2+} , this run-up does not occur. Instead, a rapid run down occurs. The "run-up" phenomenon is
30 likely due to calcium chelation inside the cells. T type Ca^{2+} currents do not show this effect.

Intracellular perfusion patch clamp experiments demonstrated that basal $[Ca^{2+}]_i$ regulates L type Ca^{2+} current amplitude in INS-1 cells:

Intracellular perfusion of a solution containing high Ca^{2+} (Fig. 9A) causes a substantial reduction in the L type Ca^{2+} current. L type Ca^{2+} currents were elicited by a voltage step to +10 mV from a holding potential of -80 mV. The $[Ca^{2+}]_i$ was measured directly using fura-2 ratiometric fluorescence. The effect of a high $[Ca^{2+}]_i$ (272 nM) on the IV relationship is shown in Fig. 9B. Perfusing in high $[Ca^{2+}]_i$, substantially reduces the high voltage current component, but does not affect the low current component. The high $[Ca^{2+}]_i$ caused a shift in peak current to negative voltages, and Ca^{2+} currents were enhanced at negative voltages. This effect seemed to result in a potentiation of the T type Ca^{2+} current (Fig. 9D). Slow deactivating T type Ca^{2+} currents showed a shift in activation upon perfusion of high $[Ca^{2+}]_i$. This may account for the shift in IV. Various concentrations of $[Ca^{2+}]_i$ regulated the activity of L type Ca^{2+} channels (Fig. 9C). Perfusing a low $[Ca^{2+}]_i$ from an existing high $[Ca^{2+}]_i$ (632 nM to 0 nM) caused an increase in the L type Ca^{2+} current over time, however perfusing in high $[Ca^{2+}]_i$ (0 nM to 272 nM and 0 nM to 632 nM) inhibits the L type Ca^{2+} current over time. The levels of $[Ca^{2+}]_i$ therefore have regulatory effects on both the L type Ca^{2+} current and T type Ca^{2+} current, with $[Ca^{2+}]_i$ having significant feedback regulation on the L type Ca^{2+} current.

Effect of basal $[Ca^{2+}]_i$ on Ca^{2+} influx:

The effect of basal $[Ca^{2+}]_i$ on Ca^{2+} influx was examined using the Ca^{2+} dye indicator fura-2 and fluorescence measurements. Voltage-dependent Ca^{2+} influx in a single cell was obtained by perfusion of an

osmotically balanced solution containing 50 mM KCl into the recording chamber. Voltage-dependent increases in $[Ca^{2+}]_i$ occur primarily through nifedipine sensitive Ca^{2+} channels. The resting basal $[Ca^{2+}]_i$ in INS-1 cells was approximately 60-80 nM under the experimental conditions. $[Ca^{2+}]_i$ was determined by a standard curve obtained from a fura-2 calcium imaging kit (Molecule Probes). The empirical K_d obtained for calcium binding to fura-2 in the system was 296 ± 20 nM. When basal $[Ca^{2+}]_i$ remains low, subsequent voltage stimulation with 50 KCl induces rapid and large calcium influx into the cell and these calcium changes are stereotyped upon repetitive stimulation when basal calcium is restored (Fig. 10). In this experiment, following the 50 KCl depolarization, the cell was repolarized by perfusion of the original 5 mM KCl solution. After repolarization, basal $[Ca^{2+}]_i$ slowly reset and then a second 50 KCl depolarization induced a similar $[Ca^{2+}]_i$ transient. When the basal calcium is not allowed to reset, a defect in the second voltage induced calcium transient occurs (Fig. 11). In this experiment, after repolarization, the second depolarization was applied before basal $[Ca^{2+}]_i$ could return to its original value, and thus, the $[Ca^{2+}]_i$ transient is substantially reduced. These findings suggest that basal $[Ca^{2+}]_i$ plays a prominent role in the regulation of voltage dependent Ca^{2+} influx in INS-1 cells. Therefore effectors of basal $[Ca^{2+}]_i$ will have important impact on the amount of calcium that can enter the cell.

Streptozotocin induced high basal $[Ca^{2+}]_i$ inhibits KCl stimulated Ca^{2+} influx:

To reiterate the importance of basal $[Ca^{2+}]_i$ on voltage stimulated Ca^{2+} influx, basal $[Ca^{2+}]_i$ in INS-1 cells was artificially enhanced by pretreating the cells

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with the toxicant, streptozotocin. Though it is known that streptozotocin induces DNA strand breaks, it has also been shown to induce Ca^{2+} channel activity in β -cells. The data shows that pretreating cells with 5 mM streptozotocin for 1 hour, followed by 3 hour recovery, causes a two-fold increase in basal calcium (Fig. 22). These cells when stimulated by 50 KCl had reduced calcium influx compared to control cells.

10

EXAMPLE V**Inhibition of T type Calcium Channel
with Mibefradil Metabolite**

It has been shown that mibefradil (Ro 40-5967) exerts a selective inhibitory effect on T-type Ca^{2+} currents, although at higher concentrations it can antagonize high voltage-activated Ca^{2+} currents. The action of mibefradil on Ca^{2+} channels is use- and steady state-dependent and the binding site of mibefradil on L-type Ca^{2+} channels is different from that of dihydropyridines. By using conventional whole-cell and perforated patch-clamp, mibefradil is shown to have an inhibitory effect on both T- and L-type Ca^{2+} currents in insulin-secreting cells. However, the effect on L-type Ca^{2+} currents was time-dependent and poorly reversible in perforated patch experiments. Using mass spectrometry it was demonstrated that mibefradil was trapped inside cells and furthermore, a metabolite of mibefradil was detected. Intracellular application of this metabolite selectively blocked the L-type Ca^{2+} current whereas mibefradil exerted no effect. This study shows that mibefradil permeates into cells and is hydrolyzed to a metabolite that blocks L-type Ca^{2+} channels specifically by acting at the inner side of the channel.

Whole-cell patch clamp and a bath perfusion system were first used to establish the dose-dependent inhibition of mibefradil on both T- and L-types of Ca^{2+} currents. The T-type Ca^{2+} current was measured at -30 mV when the membrane was held at -90 mV and the L-type current was measured at +20 mV when the membrane was held at -40 mV. The currents were measured twice at each concentration of mibefradil with 2 min in between measurements. The dose dependent inhibition of T-type Ca^{2+} current is shown in Fig. 3A. The 50% inhibitory concentration (IC_{50}) was 865 nM. No time-dependent inhibition was observed. In contrast, the inhibition of L-type Ca^{2+} currents could not be fitted with a one-to-one binding curve (Fig. 3B). Administration of 1 μM mibefradil progressively reduced L-type Ca^{2+} current up to 70% of the beginning amplitude after 10 minutes ($n = 4$), which indicated that a more complicated pharmacological mechanism was involved in the action of mibefradil on the L-type Ca^{2+} currents.

A drug diffusing system was then used to test the reversibility of the antagonism of T- and L-type Ca^{2+} currents by mibefradil. Small volumes (approximately 2 μl) of drugs were delivered in close proximity to the recording cell with a quartz capillary positioned by a micromanipulator. After administration, drugs diffused throughout the entire recording chamber containing 2 ml of bath solution. This drug diffusing system was used to test the reversibility of 30 μM of NiCl_2 on the T-type Ca^{2+} currents (Fig. 4). The amplitude of T-type current was rapidly reduced to 40% and gradually returned to 80% of the initial level within 3 minutes. Using this system, it was found that the inhibition of mibefradil on the T-type Ca^{2+} current was clearly reversible. In contrast,

the inhibition of the L-type Ca^{2+} current was poorly reversible (Fig. 4).

The poor reversibility and time-dependent inhibition of the L-type Ca^{2+} current by mibefradil suggested that this drug may have an accumulation effect over time. This hypothesis was tested by applying a very low dose of mibefradil on cells and recording the L-type Ca^{2+} currents for a long time in the perforated patch-clamp configuration. As shown in Fig. 5A, after 25 minutes of 10 nM mibefradil administration, the relative currents were reduced to 70%, whereas the currents remained unchanged for control patches. Incubation of cells with 10 nM mibefradil for two hours resulted in further reduction of current densities as recorded by perforated patches (Fig. 5B). At a concentration of 10 nM, mibefradil exhibited no long-term effect on the T-type Ca^{2+} current.

To test the hypothesis that mibefradil may permeate through the cell membrane to the cytoplasm and be trapped inside cells, the presence of mibefradil was examined in cells pre-incubated with 20 μM of mibefradil using mass spectrometry. After 3 washes, mibefradil (peaked at 496 MW) was still detected in cells (Fig. 6B). The concentration of intracellular mibefradil after one minute incubation was $3.18 \pm 0.78 \mu\text{M}$ ($n = 3$). The localization of mibefradil in cells was examined by measuring the concentration of mibefradil in the pellets and supernatants after lysis of the cells. Most of the mibefradil (92%) was detected in the supernatants and 0% was found in the pellets after washing cells with methanol, indicating that mibefradil was trapped in the cytoplasm. In addition, a peak (MW = 423) was detected which represented a hydrolyzed metabolite of mibefradil, Des-methoxyacetyl mibefradil (dm-mibefradil), which is a

major metabolite as documented previously (Wiltshire et al. 1992). By varying the time of pre-incubation, it was found that dm-mibefradil accumulated inside the cells in a time-dependent manner (Fig. 6A). This accumulation is
5 consistent with the concept that dm-mibefradil has lower — membrane permeability than its precursor mibefradil.

It was then tested whether or not mibefradil or dm-mibefradil inhibits L- or T-type Ca^{2+} currents from inside of cells. Both L- and T-type currents were
10 measured in the whole-cell patch clamp configuration when $1\ \mu\text{M}$ of mibefradil or dm-mibefradil was included in the pipette solution. As shown in Figs. 7A and 7B, intracellular application of $1\ \mu\text{M}$ mibefradil did not have inhibitory effects on either L-type or T-type Ca^{2+}
15 currents, whereas the same concentration of dm-mibefradil specifically blocked the L-type Ca^{2+} current. As the bath solution contained no drug in this series of experiments, the inhibitory effect of dm-mibefradil is considered to be acting on the inside domain of L-type Ca^{2+} channels.
20 The inhibitory effect of dm-mibefradil on T-type Ca^{2+} currents was similar to the effect of mibefradil when it was applied in the bath solution, suggesting that the methoxyacetyl group of mibefradil does not play a key role in binding to the extracellular receptor site of
25 T-type Ca^{2+} channel protein. However, this methoxyacetyl group is necessary for blocking L-type Ca^{2+} channel from the inside of cells, indicating a modification in the methoxyacetyl group of mibefradil can result in a more selective antagonist of T-type Ca^{2+} channels.

EXAMPLE VI

**LVA Ca^{2+} Current Mediates Cytokine-Induced
Pancreatic β -cell Death**

Insulin-dependent diabetes mellitus is characterized
5 by the selective destruction of pancreatic β -cells.
Chronic treatment with cytokines induced a low voltage-
activated (LVA) Ca^{2+} current in mouse β -cells. The
concomitant increase in the basal cytoplasmic free Ca^{2+}
concentration ($[\text{Ca}^{2+}]_i$) was associated with DNA
10 fragmentation and cell death. Antagonists of LVA Ca^{2+}
channels prevented this elevation of basal $[\text{Ca}^{2+}]_i$ and DNA
fragmentation, and reduced the percentage of cell death.
Exposure to cytokines did not affect the profile of Ca^{2+}
currents or basal $[\text{Ca}^{2+}]_i$ in glucagon-secreting α -cells.
15 An increased Ca^{2+} signal through LVA Ca^{2+} channels may thus
be a key feature in cytokine-induced β -cell destruction.

The effects of chronic cytokine treatment on the
voltage-sensitive Ca^{2+} currents in primary cultured mouse
islet cells was examined. After treatment with IL-1 β (25
20 U/ml) and IFN γ (300 U/ml) for 6 h, an LVA Ca^{2+} current was
induced in these cells (Fig. 23A). This current was
present in 48% of cytokine-treated mouse islet cells. No
such current was observed when the cells were treated
with either IL-1 β or IFN γ alone. Experiments were
25 conducted at different times recording LVA Ca^{2+} currents
induced by cytokines, and the results indicate that no
further increase in current density occurs even after
treatment for 48 h. This LVA current has not been
observed in non-treated cells. The steady state
30 inactivation curve of the cytokine-induced LVA Ca^{2+}
currents displayed a low voltage property (Fig. 23E)
similar to the inactivation curve of the LVA currents in
NOD mouse islets cells. This current was also blocked by
NiCl $_2$ (10 μM ; n = 4; Fig. 23F). It has been reported that

low concentration of NiCl_2 selectively block LVA current, a profound increase in Ca^{2+} current density was observed over the voltages between -20 and 20 mV. These high voltage-activated Ca^{2+} currents are nifedipine sensitive currents (completely blocked by 10 μM nifedipine), and the increase in this current density is similar to the increased L type Ca^{2+} current density observed after treatment of β -cells with serum from IDDM patients.

As α -cells are more resistant to the toxic effects of cytokines than β -cells, the effects of cytokines on the Ca^{2+} currents in a glucagon-secreting cell line (α -TC1) was also examined. This cell line, like α -cells, is more resistant to the cytotoxic effect of cytokines. Treatment of α -TC1 cells with IL-1 β and IFN γ failed to induce LVA Ca^{2+} currents and did not alter the current density (Figs. 23C and 23D). Therefore, the induction of LVA Ca^{2+} currents and increased Ca^{2+} current density observed after chronic treatment with cytokines showed specificity for β -cells.

LVA Ca^{2+} channels are activated at low membrane potentials. This unique feature may allow then to regular $[\text{Ca}^{2+}]_i$ under nonstimulatory conditions. Indeed, basal $[\text{Ca}^{2+}]_i$ in cytokine-treated cells was approximately 3-fold higher than in nontreated cells (Fig. 24A). This increase in basal $[\text{Ca}^{2+}]_i$ was blocked by NiCl_2 (10 μM), but not by the L type Ca^{2+} channel antagonist, nifedipine (10 μM). Cytokines failed to increase basal $[\text{Ca}^{2+}]_i$ in α -TC1 cells (Fig. 24B). These results suggest that Ca^{2+} influx through LVA Ca^{2+} channels is responsible for the cytokine-induced elevation in basal $[\text{Ca}^{2+}]_i$ in β -cells.

It has been shown that cytokines induce apoptosis in human pancreatic islet cells. Apoptosis is also the mode of cell death in the development of IDDM in the NOD mouse and in multiple low dose streptozotocin-induced IDDM in

the mouse, and is involved in β -cell destruction. As a marker of apoptosis, DNA fragmentation has been reported to precede β -cell lysis.

β -TC3 cells, a mouse β -cell line, were used to demonstrate the role of LVA Ca^{2+} channels in cytokine-mediated DNA fragmentation. The LVA Ca^{2+} current density was first examined before and after cytokine treatment. The LVA Ca^{2+} current (at $V_m = -30$ mV) in β -TC3 cells was increased from 1.86 ± 0.33 (pA/pF; $n = 30$) to 3.45 ± 0.47 (pA/pF; $n = 10$) after treatment with cytokines (25 U/ml IL-1 β , 100 U/ml IFN γ , and 100 U/ml TNF α) for 25 h. This indicates that the LVA Ca^{2+} current in β -TC3 cells is regulated by cytokines, as seen in mouse islet cells. As shown in Fig. 24, cytokine-induced DNA fragmentation displayed a ladder pattern of oligonucleosomal fragments. The three LVA Ca^{2+} channel blockers, NiCl_2 , amiloride, and mibefradil, all independently prevented cytokine-induced DNA fragmentation. In contrast, nifedipine had not inhibitory effect on DNA fragmentation induced by cytokines. This experiment has been repeated in β -TC3 cells ($n = 2$) as well as in NIT-1 cells ($n = 3$), a β -cell line derived from NOD mice, and the same results were obtained.

The function of LVA Ca^{2+} channels in cytokine-mediated cell death in β -TC3 cells was then examined. Many cells died when the medium contained 25 U/ml IL-1 β , 100 U/ml IFN γ , and 100 U/ml TNF α ; however, NiCl_2 (20 μM) effectively reduced the β -cell killing potency of cytokines in both a time- and dose-dependent manner (Figs. 25A and 25B, respectively). In contrast, nifedipine did not exhibit a protective effect. Similar results were obtained from an experiment conducted in NIT-1 cells with mibefradil, which also reduced β -cell death induced by cytokines. These results demonstrate

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that LVA Ca^{2+} channels enhance the vulnerability of β -cells to the cytotoxic effects of cytokines.

Although preferred embodiments have been depicted
5 and described in detail herein, it will be apparent to —
those skilled in the relevant art that various
modifications, additions, substitutions and the like can
be made without departing from the spirit of the
invention and these are therefore considered to be within
10 the scope of the invention as defined in the claims which
follow.

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SEQUENCE LISTING

DNASIS DNA Translation [T-INS]
 File Name : T-INS
 Range : 2 - 7286 Mode : Normal
 Codon Table : Universal

5' *start of 560 3' + 4*

GAG	CTG	AGC	TGA	ACT	GGC	CCT	CCT	GGG	GAC	TCA	GCA	AGC	TCT	CTA	GAG	CCC	CCC	55
E	L	S	*	T	G	P	P	G	D	S	A	S	S	L	E	P	P	
ACA	TGC	TCC	CCC	ACC	GGG	GTC	CCC	CCG	TTG	CGT	GAG	GAC	ACC	TCC	TCT	GAG	GGG	109
T	C	S	P	T	G	V	P	R	L	R	E	D	T	S	S	E	G	
CTC	CGC	TCG	CCC	CTC	TTC	GGA	CCC	CCC	GGG	GCC	CCG	GCT	GGC	154	163			
L	R	S	P	L	F	G	P	P	G	A	P	A	G	Q	R	M	D	
GAG	GAG	GAG	GAT	GGA	CCG	GGC	GCC	GAG	GAG	TCG	GGA	CAG	CCC	CGT	AGC	TTC	ACG	217
E	E	E	D	G	A	G	A	E	E	S	G	Q	P	R	S	F	T	
CAG	CTC	AAC	GAC	CTG	TCC	GGG	GCC	GGG	GCC	CGG	GAG	GGG	CCG	GGG	TCG	ACG	GAA	271
Q	L	N	D	L	S	G	A	G	G	R	Q	G	P	G	S	T	E	
AAG	GAC	CCG	GGC	AGC	GCG	GAC	TCC	GAG	GCG	GAG	GGG	CTG	CCG	TAC	CCG	GCG	CTA	325
K	D	P	G	S	A	D	S	E	A	E	G	L	P	Y	P	A	L	
GCC	CCG	GTG	GTT	TTC	TTC	TAC	TTG	AGC	CAG	GAC	AGC	CGC	CCG	CGG	AGC	TGG	TGT	379
A	P	V	V	F	F	Y	L	S	Q	D	S	R	P	R	S	W	C	
CTC	CGC	ACG	GTC	TGT	AAC	CCG	TGG	TTC	GAG	CGA	GTC	AGT	ATG	CTG	GTC	ATT	CTT	433
L	R	T	V	C	N	P	W	F	E	R	V	S	M	L	V	I	L	
CTC	AAC	TGT	GTG	ACT	CTG	GGT	ATG	TTC	AGG	CCG	TGT	GAG	GAC	ATT	GCC	TGT	GAC	487
L	N	C	V	T	L	G	M	F	R	P	C	E	D	I	A	C	D	
TCC	CAG	CGC	TGC	CGG	ATC	CTG	CAG	GCC	TTC	GAT	GAC	TTC	ATC	TTT	GCC	TTC	TTT	541
S	Q	R	C	R	I	L	Q	A	F	D	D	F	I	F	A	F	F	
GCT	GTG	GAA	ATG	GTG	GTG	AAG	ATG	GTG	GCC	TTG	GGC	ATC	TTT	GGG	AAG	AAA	TGT	595
A	V	E	M	V	V	K	M	V	A	L	G	I	F	G	K	K	C	
TAC	CTG	GGA	GAC	ACT	TGG	AAC	CGG	CTT	GAC	TTT	TTC	ATT	GTC	ATT	GCA	GGG	ATG	649
Y	L	G	D	T	W	N	R	L	D	F	F	I	V	I	A	G	M	

*start of 560 ID No: 1
560 ID No: 2
(coding regions)*

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DNASIS DNA Translation [T-INS]

CTG	GAG	TAT	TCG	CTG	GAC	CTG	CAG	AAC	GTC	AGC	TTC	TCC	GCA	GTC	AGG	ACA	703	GTC
L	E	Y	S	L	D	L	Q	N	V	S	F	S	A	V	R	T	V	
CGT	GTG	CTG	CGA	CCG	CTC	AGG	GCC	ATT	AAC	CGG	GTG	CCC	AGC	ATG	CGC	ATT	712	CTC
R	V	L	R	P	L	R	A	I	N	R	V	P	S	M	R	I	L	
GTC	ACA	TTA	CTG	CTG	GAC	ACC	TTG	CCT	ATG	CTG	GGC	AAC	GTC	802	CTG	CTG	CTC	811
V	T	L	L	L	D	T	L	P	M	L	G	N	V	L	L	L	C	
TTC	TTC	GTC	TTT	TTC	ATC	TTT	GGC	ATC	GTG	GGC	GTC	CAG	CTG	TGG	GCA	GGA	CTG	820
F	F	V	F	F	I	F	G	I	V	G	V	Q	L	W	A	G	L	
CTT	CGC	AAC	CGA	TGC	TTC	CTC	CCC	GAG	AAC	TTC	AGC	CTC	CCC	CTG	AGC	GTG	GAC	874
L	R	N	R	C	F	L	P	E	N	F	S	L	P	L	S	V	D	
CTG	GAG	CCT	TAT	TAC	CAG	ACA	GAG	AAT	GAG	GAC	GAG	AGC	CCC	TTC	ATC	TGC	TCT	928
L	E	P	Y	Y	Q	T	E	N	E	D	E	S	P	F	I	C	S	
CAG	CCT	CGG	GAG	AAT	GGC	ATG	AGA	TCC	TGC	AGG	AGT	GTG	CCC	ACA	CTG	CGT	GGG	982
Q	P	R	E	N	G	M	R	S	C	R	S	V	P	T	L	R	G	
GAA	GGC	GGT	GGT	GGC	CCA	CCC	TGC	AGT	CTG	GAC	TAT	GAG	ACC	TAT	AAC	AGT	TCC	1036
E	G	G	G	G	P	P	C	S	L	D	Y	E	T	Y	N	S	S	
AGC	AAC	ACC	ACC	TGT	GTC	AAC	TGG	AAC	CAG	TAC	TAT	ACC	AAC	TGC	TCT	GCG	GGC	1090
S	N	T	T	C	V	N	W	N	Q	Y	Y	T	N	C	S	A	G	
GAG	CAC	AAC	CCC	TTC	AAA	GGC	GCC	ATC	AAC	TTT	GAC	AAC	ATT	GGC	TAT	GCC	TGG	1144
E	H	N	P	F	K	G	A	I	N	F	D	N	I	G	Y	A	W	
ATC	GCC	ATC	TTC	CAG	GTC	ATC	ACA	CTG	GAG	GGC	TGG	GTC	GAC	ATC	ATG	TAC	TTC	1198
I	A	I	F	Q	V	I	T	L	E	G	W	V	D	I	M	Y	F	
GTA	ATG	GAC	GCT	CAC	TCC	TTC	TAC	AAC	TTC	ATC	TAC	TTC	ATT	CTT	CTC	ATC	ATC	1252
V	M	D	A	H	S	F	Y	N	F	I	Y	F	I	L	L	I	I	
GTG	GGC	TCC	TTC	TTC	ATG	ATC	AAC	CTG	TGC	CTG	GTG	GTG	ATT	GCC	ACG	CAG	TTC	1306
V	G	S	F	F	M	I	N	L	C	L	V	V	I	A	T	Q	F	

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DNASIS DNA Translation (T-INS)

1360	1369	1378	1387	1396	1405
TCC GAG ACC	AAA CAG CGG	GAG AGT CAG	CTG ATG CGG	GAG CAG CGT	GTA CGA TTC
S E T	K Q R	E S Q	L M R	E Q R	V R F
1414	1423	1432	1441	1450	1459
CTG TCC AAT	GCT AGC ACC	CTG GCA AGC	TTC TCT GAG	CCA GGC AGC	TGC TAT GAG
L S N	A S T	L A S	F S E	P G S	C Y E
1468	1477	1486	1495	1504	1513
GAG CTA CTC	AAG TAC CTG	GTG TAC ATC	CTC CGA AAA	GCA GCC CGA	AGG CTG GCC
E L L	K Y L	V Y I	L R K	A A R	R L A
1522	1531	1540	1549	1558	1567
CAG GTC TCT	AGG GCT ATA	GGC GTG CGG	GCT GGG CTG	CTC AGC AGC	CCA GTG GCC
Q V S	R A I	G V R	A G L	L S S	P V A
1576	1585	1594	1603	1612	1621
CGT AGT GGG	CAG GAG CCC	CAG CCC AGT	GGC AGC TGC	ACT CGC TCA	CAC CGT CGT
R S G	Q E P	Q P S	G S C	T R S	H R R
1630	1639	1648	1657	1666	1675
CTG TCT GTC	CAC CAC CTG	GTC CAC CAC	CAT CAC CAC	CAC CAT CAC	CAC TAC CAC
L S V	H H L	V H H	H H H	H H H	H Y H
1684	1693	1702	1711	1720	1729
CTG GGT AAT	GGG ACG CTC	AGA GTT CCC	CGG GCC AGC	CCA GAG ATC	CAG GAC AGG
L G N	G T L	R V P	R A S	P E I	Q D R
1738	1747	1756	1765	1774	1783
GAT GCC AAT	GGG TCT CGC	CGG CTC ATG	CTA CCA CCA	CCC TCT ACA	CCC ACT CCC
D A N	G S R	R L M	L P P	P S T	P T P
1792	1801	1810	1819	1828	1837
TCT GGG GGC	CCT CCG AGG	GGT GCG GAG	TCT GTA CAC	AGC TTC TAC	CAT GCT GAC
S G G	P P R	G A E	S V H	S F Y	H A D
1846	1855	1864	1873	1882	1891
TGC CAC TTG	GAG CCA GTC	CGT TGC CAG	GCA CCC CCT	CCC AGA TGC	CCA TCG GAG
C H L	E P V	R C Q	A P P	P R C	P S E
1900	1909	1918	1927	1936	1945
GCA TCT GGT	AGG ACT GTG	GGT AGT GGG	AAG GTG TAC	CCC ACT GTG	CAT ACC AGC
A S G	R T V	G S G	K V Y	P T V	H T S
1954	1963	1972	1981	1990	1999
CCT CCA CCA	GAG ATA CTG	AAG GAT AAA	GCA CTA GTG	GAG GTG GCC	CCC AGC CCT
P P P	E I L	K D K	A L V	E V A	P S P
2008	2017	2026	2035	2044	2053
GGG CCC CCC	ACC CTC ACC	AGC TTC AAC	ATC CCA CCT	GGG CCC TTC	AGC TCC ATG
G P P	T L T	S F N	I P P	G P F	S S M

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DNASTIS DNA Translation [T-INS]

2062	2071	2080	2089	2098	2107
CAC AAG CTC	CTG GAG ACA	CAG AGT ACG	GGA GCC TGC	CAT AGC TCC	TGC AAA ATC
H K L	L E T	Q S T	G A C	H S S	C K I
2116	2125	2134	2143	2152	2161
TCC AGC CCT	TGC TCC AAG	GCA GAC AGT	GGA GCC TGC	GGG CCG GAC	AGT TGT CCC
S S P	C S K	A D S	G A C	G P D	S C P
2170	2179	2188	2197	2206	2215
TAC TGT GCC	CGG ACA GGA	GCA GGA GAG	CCA GAG TCC	GCT GAC CAT	GTC ATG CCT
Y C A	R T G	A G E	P E S	A D H	V M P
2224	2233	2242	2251	2260	2269
GAC TCA GAC	AGC GAG GCT	GTG TAT GAG	TTC ACA CAG	GAC GCT CAG	CAC AGT GAC
D S D	S E A	V Y E	F T Q	D A Q	H S D
2278	2287	2296	2305	2314	2323
CTC CGG GAT	CCC CAC AGC	CGG CGG CGA	CAG CGG AGC	CTG GGC CCA	GAT GCA GAG
L R D	P H S	R R R	Q R S	L G P	D A E
2332	2341	2350	2359	2368	2377
CCT AGT TCT	GTG CTG GCT	TTC TGG AGG	CTG ATC TGT	GAC ACA TTC	CGG AAG ATC
P S S	V L A	F W R	L I C	D T F	R K I
2386	2395	2404	2413	2422	2431
GTA GAT AGC	AAA TAC TTT	GGC CGG GGA	ATC ATG ATC	GCC ATC CTG	GTC AAT ACA
V D S	K Y F	G R G	I M I	A I L	V N T
2440	2449	2458	2467	2476	2485
CTC AGC ATG	GGC ATC GAG	TAC CAC GAG	CAG CCC GAG	GAG CTC ACC	AAC GCC CTG
L S M	G I E	Y H E	Q P E	E L T	N A L
2494	2503	2512	2521	2530	2539
GAA ATC AGC	AAC ATC GTC	TTC ACC AGC	CTC TTC GCC	TTG GAG ATG	CTG CTG AAA
E I S	N I V	F T S	L F A	L E M	L L K
2548	2557	2566	2575	2584	2593
CTG CTT GTC	TAC GGT CCC	TTT GGC TAC	ATT AAG AAT	CCC TAC AAC	ATC TTT GAT
L L V	Y G P	F G Y	I K N	P Y N	I F D
2602	2611	2620	2629	2638	2647
GGT GTC ATT	GTG GTC ATC	AGT GTG TGG	GAG ATT GTG	GGC CAG CAG	GGA GGT GGC
G V I	V V I	S V W	E I V	G Q Q	G G G
2656	2665	2674	2683	2692	2701
CTG TCG GTG	CTG CGG ACC	TTC CGC CTG	ATG CGG GTG	CTG AAG CTG	GTG CGC TTC
L S V	L R T	F R L	M R V	L K L	V R F
2710	2719	2728	2737	2746	2755
CTG CCG GCC	CTG CAG CGC	CAG CTC GTG	GTG CTC ATG	AAG ACC ATG	GAC AAC GTG
L P A	L Q R	Q L V	V L M	K T M	D N V

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DNASIS DNA Translation [T-INS]

2764	2773	2782	2791	2800	2809
GCC ACC TTC	TGC ATG CTC	CTC ATG CTG	TTC ATC TTC	ATC TTC AGC	ATC CTG GGC
A T F	C M L	L M L	F I F	I F S	I L G
2818	2827	2836	2845	2854	2863
ATG CAT CTC	TTT GGT TGC	AAG TTC GCA	TCT GAA CCG	GAT GGG GAC	ACG TTG CCA
M H L	F G C	K F A	S E R	D G D	T L P
2872	2881	2890	2899	2908	2917
GAC CGG AAG	AAT TTC GAC	TCC CTG CTC	TGG GCC ATC	GTC ACT GTC	TTT CAG ATT
D R K	N F D	S L L	W A I	V T V	F Q I
2926	2935	2944	2953	2962	2971
CTG ACT CAG	GAA GAC TGG	AAT AAA GTC	CTC TAC AAC	GGC ATG GCC	TCC ACA TCG
L T Q	E D W	N K V	L Y N	G M A	S T S
2980	2989	2998	3007	3016	3025
TCT TGG GCT	GCT CTT TAC	TTC ATC GCC	CTC ATG ACT	TTT GGC AAC	TAT GTG CTC
S W A	A L Y	F I A	L M T	F G N	Y V L
3034	3043	3052	3061	3070	3079
TTT AAC CTG	CTG GTG GCC	ATT CTT GTG	GAA GGA TTC	CAG GCA GAG	GAA ATC GGC
F N L	L V A	I L V	E G F	Q A E	E I G
3088	3097	3106	3115	3124	3133
AAA CCG GAA	GAT GCG AGT	GGA CAG TTA	AGC TGT ATT	CAG CTG CCT	GTC AAC TCT
K R E	D A S	G Q L	S C I	Q L P	V N S
3142	3151	3160	3169	3178	3187
CAG GGG GGA	GAT GCC ACC	AAG TCT GAG	TCA GAG CCT	GAT TTC TTT	TCG CCC AGT
Q G G	D A T	K S E	S E F	D F F	S P S
3196	3205	3214	3223	3232	3241
GTG GAT GGT	GAT GGG GAC	AGA AAG AAG	CGC TTG GCC	CTG GTG GCT	TTG GGA GAA
V D G	D G D	R K K	R L A	L V A	L G E
3250	3259	3268	3277	3286	3295
CAC GCG GAA	CTA CGA AAG	AGC CTT TTG	CCA CCC CTC	ATC ATC CAT	ACG GCT GCG
H A E	L R K	S L L	P P L	I I H	T A A
3304	3313	3322	3331	3340	3349
ACA CCA ATG	TCA CTA CCC	AAG AGC TCC	AGC ACA GGT	GTG GGG GAA	GCA CTG GGC
T P M	S L P	K S S	S T G	V G E	A L G
3358	3367	3376	3385	3394	3403
TCT GGC TCT	CGA CGT ACC	AGT AGC AGT	GGG TCC GCT	GAG CCT GGA	GCT GCC CAC
S G S	R R T	S S S	G S A	E P G	A A H
3412	3421	3430	3439	3448	3457
CAT GAG ATG	AAA TCT CCG	CCA AGT GCC	CGC AGC TCC	CCG CAC AGT	CCC TGG AGT
H E M	K S P	P S A	R S S	P H S	P W S

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DNASIS DNA Translation [T-INS]

3466	3475	3484	3493	3502	3511
GCG GCA AGC AGC TGG ACC AGC AGG CGC TCC AGC AGG AAC AGC CTG GGC CGG GCC					
A A S S W T S R R S S R N S L G R A					
3520	3529	3538	3547	3556	3565
CCC AGC CTA AAG CGG AGG AGC CCG AGC GGG GAG CGG AGG TCC CTG CTG TCT GGA					
P S L K R R S P S G E R R S L L S G					
3574	3583	3592	3601	3610	3619
GAG GGC CAG GAG AGT CAG GAT GAG GAG GAA AGT TCA GAA GAG GAC CGG GCC AGC					
E G Q E S Q D E E E S S E E D R A S					
3628	3637	3646	3655	3664	3673
CCA GCA GGC AGT GAC CAT CGC CAC AGG GGT TCC TTG GAA CGT GAG GCC AAG AGT					
P A G S D H R H R G S L E R E A K S					
3682	3691	3700	3709	3718	3727
TCC TTT GAC CTG CCT GAC ACT CTG CAG GTG CCG GGG CTG CAC CGC ACA GCC AGC					
S F D L P D T L Q V P G L H R T A S					
3736	3745	3754	3763	3772	3781
GGC CGG AGC TCT GCC TCT GAG CAC CAA GAC TGT AAT GGC AAG TCG GCT TCA GGG					
G R S S A S E H Q D C N G K S A S G					
3790	3799	3808	3817	3826	3835
CGT TTG GCC CGC ACC CTG AGG ACT GAT GAC CCC CAA CTG GAT GGG GAT GAT GAC					
R L A R T L R T D D P Q L D G D D D					
3844	3853	3862	3871	3880	3889
AAT GAT GAG GGA AAT CTG AGC AAA GGG GAA CGC ATA CAA GCC TGG GTC AGA TCC					
N D E G N L S K G E R I Q A W V R S					
3898	3907	3916	3925	3934	3943
CGG CTT CCT GCC TGT TGC CGA GAG CGA GAT TCC TGG TCG GCC TAT ATC TTT CCT					
R L P A C C R E R D S W S A Y I F P					
3952	3961	3970	3979	3988	3997
CCT CAG TCA AGG TTT CGT CTC CTG TGT CAC CGG ATC ATC ACC CAC AAG ATG TTT					
P Q S R F R L L C H R I I T H K M F					
4006	4015	4024	4033	4042	4051
GAC CAT GTG GTC CTC GTC ATC ATC TTC CTC AAC TGT ATC ACC ATC GCT ATG GAG					
D H V V L V I I F L N C I T I A M E					
4060	4069	4078	4087	4096	4105
CGC CCC AAA ATT GAC CCC CAC AGC GCT GAG CGC ATC TTC CTG ACC CTC TCC AAC					
R P K I D P H S A E R I F L T L S N					
4114	4123	4132	4141	4150	4159
TAC-ATC TTC ACG GCA GTC TTT CTA GCT GAA ATG ACA GTG AAG GTG GTG GCA CTG					
Y I F T A V F L A E M T V K V V A L					

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DNASIS DNA Translation [T-INS]

4168	4177	4186	4195	4204	4213
GGC TGG TGC TTT	GGG GAG CAG	GCC TAC CTG	CGC AGC AGC	TGG AAT GTG	CTG GAC
G W C F	G E Q	A Y L	R S S	W N V	L D
4222	4231	4240	4249	4258	4267
GGC TTG CTG GTG	CTC ATC TCC	GTC ATC GAC	ATC CTG GTC	TCC ATG GTC	TCC GAC
G L L V	L I S	V I D	I L V	S M V	S D
4276	4285	4294	4303	4312	4321
AGC GGC ACC AAG	ATC CTT GGC	ATG CTG AGG	GTG CTG CGG	CTG CTG CGG	ACC CTG
S G T K	I L G	M L R	V L R	L L R	T L
4330	4339	4348	4357	4366	4375
CGT CCA CTC AGG	GTC ATC AGC	CGG GCC CAG	GGA CTG AAG	CTG GTG GTA	GAG ACT
R P L R	V I S	R A Q	G L K	L V V	E T
4384	4393	4402	4411	4420	4429
CTG ATG TCA TCC	CTC AAA CCC	ATT GGC AAC	ATT GTG GTC	ATT TGC TGT	GCC TTC
L M S S	L K P	I G N	I V V	I C C	A F
4438	4447	4456	4465	4474	4483
TTC ATC ATT TTT	GGA ATT CTC	GGG GTG CAG	CTC TTC AAA	GGG AAG TTC	TTC GTG
F I I F	G I L	G V Q	L F K	G K F	F V
4492	4501	4510	4519	4528	4537
TGT CAG GGT GAG	GAC ACC AGG	AAC ATC ACT	AAC AAA TCC	GAC TGC GCT	GAG GCC
C Q G E	D T R	N I T	N K S	D C A	E A
4546	4555	4564	4573	4582	4591
AGC TAC CGA TGG	GTC CGG CAC	AAG TAC AAC	TTT GAC AAC	CTG GGC CAG	GCT CTG
S Y R W	V R H	K Y N	F D N	L G Q	A L
4600	4609	4618	4627	4636	4645
ATG TCC CTG TTT	GTG CTG GCC	TCC AAG GAT	GGT TGG GTT	GAC ATC ATG	TAT GAT
M S L F	V L A	S K D	G W V	D I M	Y D
4654	4663	4672	4681	4690	4699
GGG CTG GAT GCT	GTG GGT GTG	GAT CAG CAG	CCC ATC ATG	AAC CAC AAC	CCC TGG
G L D A	V G V	D Q Q	P I M	N H N	P W
4708	4717	4726	4735	4744	4753
ATG CTG CTA TAC	TTC ATC TCC	TTC CTC CTC	ATC GTG GCC	TTC TTT GTC	CTG AAC
M L L Y	F I S	F L L	I V A	F F V	L N
4762	4771	4780	4789	4798	4807
ATG TTT GTG GGC	GTG GTG GTG	GAG AAC TTC	CAT AAG TGC	AGA CAG CAC	CAG GAG
M F V G	V V V	E N F	H K C	R Q H	Q E
4816	4825	4834	4843	4852	4861
GAG GAG GAG GCG	AGG CGG CGT	GAG GAG AAG	CGA CTA CGG	AGG CTG GAG	AAA AAG
E E E A	R R R	E E K	R L R	R L E	K K

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DNASIS DNA Translation [T-INS]

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      4870      4879      4888      4897      4906      4915
AGA AGG AAT CTA ATG TTG GAC GAT GTA ATT GCT TCC GGC AGC TCA GCC AGC GCT
---
R  R  N  L  M  L  D  D  V  I  A  S  G  S  S  A  S  A

      4924      4933      4942      4951      4960      4969
GCG TCA GAA GCC CAG TGC AAG CCC TAC TAC TCT GAC TAC TCG AGA TTC CGG CTC
---
A  S  E  A  Q  C  K  P  Y  Y  S  D  Y  S  R  F  R  L

      4978      4987      4996      5005      5014      5023
CTT GTC CAC CAC CTG TGT ACC AGC CAC TAC CTG GAC CTC TTC ATC ACT GGT GTC
---
L  V  H  H  L  C  T  S  H  Y  L  D  L  F  I  T  G  V

      5032      5041      5050      5059      5068      5077
ATC GGG CTG AAC GTG GTC ACT ATG GCC ATG GAA CAT TAC CAG CAG CCC CAG ATC
---
I  G  L  N  V  V  T  M  A  M  E  H  Y  Q  Q  P  Q  I

      5086      5095      5104      5113      5122      5131
CTG GAC GAG GCT CTG AAG ATC TGC AAT TAC ATC TTT ACC GTC ATC TTT GTC TTT
---
L  D  E  A  L  K  I  C  N  Y  I  F  T  V  I  F  V  F

      5140      5149      5158      5167      5176      5185
GAG TCA GTT TTC AAA CTT GTG GCC TTT GGC TTC CGC CGT TTC TTC CAG GAC AGG
---
E  S  V  F  K  L  V  A  F  G  F  R  R  F  F  Q  D  R

      5194      5203      5212      5221      5230      5239
TGG AAC CAG CTG GAC CTG GCT ATT GTG CTT CTG TCC ATC ATG GGC ATC ACA CTG
---
W  N  Q  L  D  L  A  I  V  L  L  S  I  M  G  I  T  L

      5248      5257      5266      5275      5284      5293
GAG GAG ATT GAG GTC AAT GCT TCG CTG CCC ATC AAC CCC ACC ATC ATC CGT ATC
---
E  E  I  E  V  N  A  S  L  P  I  N  P  T  I  I  R  I

      5302      5311      5320      5329      5338      5347
ATG AGG GTG CTC CGC ATT GCT CGA GTT CTG AAG CTG TTG AAG ATG GCT GTG GGC
---
M  R  V  L  R  I  A  R  V  L  K  L  L  K  M  A  V  G

      5356      5365      5374      5383      5392      5401
ATG CGG GCA CTG CTG GAC ACG GTG ATG CAG GCC CTG CCC CAG GTG GGG AAC CTG
---
M  R  A  L  L  D  T  V  M  Q  A  L  P  Q  V  G  N  L

      5410      5419      5428      5437      5446      5455
GGA CTT CTC TTC ATG TTA TTG TTT TTC ATC TTT GCA GCT CTG GGC GTG GAG CTC
---
G  L  L  F  M  L  L  F  F  I  F  A  A  L  G  V  E  L

      5464      5473      5482      5491      5500      5509
TTT GGA GAC CTG GAG TGT GAT GAG ACA CAC CCT TGT GAG GGC TTG GGT CGG CAT
---
F  G  D  L  E  C  D  E  T  H  P  C  E  G  L  G  R  H

      5518      5527      5536      5545      5554      5563
GCC ACC TTT AGG AAC TTT GGT ATG GCC TTT CTG ACC CTC TTC CGA GTC TCC ACT
---
A  T  F  R  N  F  G  M  A  F  L  T  L  F  R  V  S  T

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DNASIS DNA Translation [T-INS]

5572	5581	5590	5599	5608	5617
GGT GAC AAC TGG AAT GGT ATT ATG AAG GAC ACC CTC CGG GAC TGT GAC CAG GAG					
G D N W N G I M K D T L R D C D Q E					
5626	5635	5644	5653	5662	5671
TCC ACC TGC TAC AAC ACT GTC ATC TCC CCT ATC TAC TTT GTG TCC TTC GTG CTG					
S T C Y N T V I S P I Y F V S F V L					
5680	5689	5698	5707	5716	5725
ACG GCC CAG TTT GTG CTG GTC AAC GTG GTC ATA GCT GTG CTG ATG AAG CAC CTG					
T A Q F V L V N V V I A V L M K H L					
5734	5743	5752	5761	5770	5779
GAA GAA AGC AAC AAA GAG GCC AAG GAG GAG GCC GAG CTC GAG GCC GAG CTG GAG					
E E S N K E A K E E A E L E A E L E					
5788	5797	5806	5815	5824	5833
CTG GAG ATG AAG ACG CTC AGC CCG CAG CCC CAC TCC CCG CTG GGC AGC CCC TTC					
L E M K T L S P Q P H S P L G S P F					
5842	5851	5860	5869	5878	5887
CTC TGG CCC GGG GTG GAG GGT GTC AAC AGT CCT GAC AGC CCT AAG CCT GGG GCT					
L W P G V E G V N S P D S P K P G A					
5896	5905	5914	5923	5932	5941
CCA CAC ACC ACT GCC CAC ATT GGA GCA GCC TCG GGC TTC TCC CTT GAG CAC CCC					
P H T T A H I G A A S G F S L E H P					
5950	5959	5968	5977	5986	5995
ACG ATG GTA CCC CAC CCC GAG GAG GTG CCA GTC CCC CTA GGA CCA GAC CTG CTG					
T M V P H P E E V P V P L G P D L L					
6004	6013	6022	6031	6040	6049
ACT GTG AGG AAG TCT GGT GTC AGC CGG ACG CAC TCT CTG CCC AAT GAC AGC TAC					
T V R K S G V S R T H S L P N D S Y					
6058	6067	6076	6085	6094	6103
ATG TGC CGC AAT GGG AGC ACT GCT GAG AGA TCC CTA GGA CAC AGG GGC TGG GGG					
M C R N G S T A E R S L G H R G W G					
6112	6121	6130	6139	6148	6157
CTC CCC AAA GCC CAG TCA GGC TCC ATC TTG TCC GTT CAC TCC CAA CCA GCA GAC					
L P K A Q S G S I L S V H S Q P A D					
6166	6175	6184	6193	6202	6211
ACC AGC TGC ATC CTA CAG CTT CCC AAA GAT GTG CAC TAT CTG CTC CAG CCT CAT					
T S C I L Q L P K D V H Y L L Q P H					
6220	6229	6238	6247	6256	6265
GGG GCC CCC ACC TGG GGC GCC ATC CCT AAA CTA CCC CCA CCT GGC CGC TCC CCT					
G A P T W G A I P K L P P P G R S P					

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DNASIS DNA Translation [T-INS]

6274	6283	6292	6301	6310	6319
CTG GCT CAG AGG	CCT CTC AGG	CGC CAG GCA	GCA ATA AGG	ACT GAC TCC	CTG GAT
L A Q R	P L R	R Q A	A I R	T D S	L D
6328	6337	6346	6355	6364	6373
GTG CAG GGC	CTG GGT AGC	CGG GAA GAC	CTG TTG TCA	GAG GTG AGT	GGG CCC TCC
V Q G L	G S R	E D L	L S E	V S G	P S
6382	6391	6400	6409	6418	6427
TGC CCT CTG	ACC CGG TCC	TCA TCC TTC	TGG GGC GGG	TGC AGC ATC	CAG GTG CAG
C P L T	R S S	S F W	G G S	S I Q	V Q
6436	6445	6454	6463	6472	6481
CAG CGT TCC	GGC ATC CAG	AGC AAA GTC	TCC AAG CAC	ATC CGC CTG	CCA GCC CCT
Q R S G	I Q S	K V S	K H I	R L P	A P
6490	6499	6508	6517	6526	6535
TGC CCA GGC	CTG GAA CCC	AGC TGG GCC	AAG GAC CCT	CCA GAG ACC	AGA AGC AGC
C P G L	E P S	W A K	D P P	E T R	S S
6544	6553	6562	6571	6580	6589
TTA GAG CTG	GAC ACG GAG	CTG AGC TGG	ATT TCA GGA	GAC CTC CTT	CCC AGC AGC
L E L D	T E L	S W I	S G D	L L P	S S
6598	6607	6616	6625	6634	6643
CAG GAA GAA	CCC CTG TCC	CCA CGG GAC	CTG AAG AAG	TGC TAC AGT	GTA GAG ACC
Q E E P	L S P	R D L	K K C	Y S V	E T
6652	6661	6670	6679	6688	6697
CAG AGC TGC	AGG CGC AGG	CCT GGG TCC	TGG CTA GAT	GAA CAG CGG	AGA CAC TCC
Q S C R	R R P	G S W	L D E	Q R R	H S
6706	6715	6724	6733	6742	6751
ATT GCT GTC	AGC TGT CTG	GAC AGC GGC	TCC CAA CCC	CGC CTA TGT	CCA AGC CCC
I A V S	C L D	S G S	Q P R	L C P	S P
6760	6769	6778	6787	6796	6805
TCA AGC CTC	GGG GGC CAA	CCT CTT GGG	GGT CCT GGG	AGC CGG CCT	AAG AAA AAA
S S L G	G Q P	L G G	P G S	R P K	K K
6814	6823	6832	6841	6850	6859
CTC AGC CCA	CCC AGT ATC	TCT ATA GAC	CCC CCG GAG	AGC CAG GGC	TCT CGG CCC
L S P P	S I S	I D P	P P E	S Q G	S R P
6868	6877	6886	6895	6904	6913
CCA TGC AGT	CCT GGT GTC	TGC CTC AGG	AGG AGG GCG	CCG GCC AGT	GAC TCT AAG
P C S P	G V C	L R R	R A P	A S D	S K
6922	6931	6940	6949	6958	6967
GAT CCC TCG	GTC TCC AGC	CCC CTT GAC	AGC ACG GCT	GCC TCA CCC	TCC CCA AAG
D P S V	S S P	L D S	T A A	S P S	P K

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DNASIS DNA Translation [T-INS]

6976	6985	6994	7003	7012	7021
AAA GAC ACG CTG AGT CTC TCT GGT TTG TCT TCT GAC CCA ACA GAC ATG GAC CCC					
K D T L S L S G L S S D P T D M D P					
7030	7039	7048	7057	7066	7075
TGA GTC CTA CCC ACT CTC CCC CAT CAC CTT TCT CCA CCG GGT GCA GAT CCT AGC					
* V L P T L P H H L S P P G A D P S					
7084	7093	7102	7111	7120	7129
TCC GCC TCC TGG GCA GCG TTT CTG AAA AGT CCC ACG TAA GCA GCA AGC AGC CAC					
S A S W A A F L K S P T * A A S S H					
7138	7147	7156	7165	7174	7183
GAG GCA CCT CAC CTG CCT TCT TCA GTG GCT GGT GGG GAT GAC GAG CAG AAC TTC					
E A P H L P S S V A G G D D E Q N F					
7192	7201	7210	7219	7228	7237
CGG AGA GTC GAT CTG AAG AGA ACA CAG CCC TGG AGC CCC TGC CTC CGG GAA GAA					
R R V D L K R T Q P W S P C L R E E					
7246	7255	7264	7273	7282	
GGA AAA GGA GAA AGC CCA GTG TGG CCA AGG CTC CCG ACA CCA GGA GCT G/3'					
G K G E S P V W P R L P T P G A/2' 560 1 + 3					
					2 + 4

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10 20 30 40 50 60
 GEAGCTGAGC TGAAGTGGCC CTCCTGGGGA CTCAGCAAGC TCTCTAGAGC CCCCCACATG
 70 80 90 100 110 120
 CTCCCCCACC GGGGTCCCCC GGTTCGGTGA GGACACCTCC TCTGAGGGGC TCCGCTCGCC
 130 140 150 160 170 180
 CCTCTTCGGA CCCCCCGGGG CCCCAGGCTGG CCAGAGGATG GACGAGGAGG AGGATGGAGC
 190 200 210 220 230 240
 GGGCGCCGAG GAGTCGGGAC AGCCCCGTAG CTTCAAGCAG CTCAACGACC TGTCGGGGGC
 250 260 270 280 290 300
 CGGGGGCCCG CAGGGGCCCG GGTGACCGA AAAGGACCCG GGCAGCGCGG ACTCCGAGGC
 310 320 330 340 350 360
 GGAGGGGGCTG CCGTACCCCG CGCTAGCCCC GGTGGTTTTC TTCTACTTGA GCCAGGACAG
 370 380 390 400 410 420
 CCGCCCCCGG AGCTGGTGTC TCCGCACGGT CTGTAACCCG TGGTTCGAGC GAGTCAGTAT
 430 440 450 460 470 480
 GCTGGTCATT CTTCTCAACT GTGTGACTCT GGGTATGTTT AGGCCGTGTG AGGACATGTC
 490 500 510 520 530 540
 CTGTGACTCC CAGCGCTGCC GGATCCTGCA GGCCTTCGAT GACTTCATCT TTGCCTTCCT
 550 560 570 580 590 600
 TGCTGTGGAA ATGGTGGTGA AGATGGTGGC CTTGGGCATC TTGGGGAAGA AATGTTACCT
 610 620 630 640 650 660
 GGGAGACACT TGGAACCGGC TTGACTTTTT CATTGTCATT GCAGGGATGC TGGAGTATTC
 670 680 690 700 710 720
 GCTGGACCTG CAGAACGTCA GCTTCTCCGC AGTCAGGACA GTCCGTGTGC TGGACCGCT
 730 740 750 760 770 780
 CAGGGCCATT AACCGGGTGC CCAGCATGCG CATTCTCGTC ACATTACTGC TGGACACCTT
 790 800 810 820 830 840
 GCCTATGCTG GGCAACGTCC TGCTGCTCTG TTTCTTGGTC TTTTTCATCT TTGGCATCGT
 850 860 870 880 890 900
 GGGCGTCCAG CTGTGGGCAG GACTGCTTCG CAACCGATGC TTCCTCCCCG AGAACTTCAG
 910 920 930 940 950 960
 CCTCCCCCTG AGCGTGGACC TGGAGCCTTA TTACCAGACA GAGAATGAGG ACGAGAGCCC
 970 980 990 1000 1010 1020
 CTTCATCTGC TCTCAGCCTC GGGAGAATGG CATGAGATCC TGCAGGAGTG TGCCACACT
 1030 1040 1050 1060 1070 1080
 GCGTGGGGAA GCGGTGGTG GCCCACCCTG CAGTCTGGAC TATGAGACCT ATAACAGTTC
 1090 1100 1110 1120 1130 1140
 CAGCAACACC ACCTGTGTCA ACTGGAACCA GTACTATACC AACTGCTCTG CGGGCGAGCA
 1150 1160 1170 1180 1190 1200
 CAACCCCTTC AAAGCGGCCA TCAACTTGA CAACATGGC TATGCCCTGA TCGCCATCTT
 1210 1220 1230 1240 1250 1260
 CCAGGTATC AACTGGAGG GCTGGGTCGA CATCATGTAC TTCGTAATGG ACGCTCACTC

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DNASIS
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1270	1280	1290	1300	1310	1320
CTTCTACAAC	TTCATCTACT	TCATTCTTCT	CATCATCGTG	GGCTCCTTCT	TCATGATCAA
1330	1340	1350	1360	1370	1380
CCTGTGCCTG	GTGGTGATTG	CCACGCAGTT	CTCCGAGACC	AAACAGCGGG	AGAGTCAGCT
1390	1400	1410	1420	1430	1440
GATGCGGGAG	CAGCGTGTAC	GATTCCCTGTC	CAATGCTAGC	ACCCTGGCAA	GCTTCTCTGA
1450	1460	1470	1480	1490	1500
CCCAGGCAGC	TGCTATGAGG	AGCTACTCAA	GTACCTGGTG	TACATCCTCC	GAAAAGCAGC
1510	1520	1530	1540	1550	1560
CCGAAGGCTG	GCCCAGGTCT	CTAGGGCTAT	AGGCGTGCGG	GCTGGGGCTGC	TCAGCAGCCC
1570	1580	1590	1600	1610	1620
AGTGGCCCGT	AGTGGGCAGG	AGCCCCAGCC	CAGTGGCAGC	TGCACTCGCT	CACACCCTCG
1630	1640	1650	1660	1670	1680
TCTGTCTGTC	CACCACCTGG	TCCACCACCA	TCACCACCAC	CATCACCCT	ACCACCTGGG
1690	1700	1710	1720	1730	1740
TAATGGGACG	CTCAGAGTTC	CCCGGGCCAG	CCCAGAGATC	CAGGACAGGG	ATGCCAATGG
1750	1760	1770	1780	1790	1800
GTCTCGCGGG	CTCATGTCTAC	CACCACCCTC	TACACCCACT	CCCTCTGGGG	GCCCTCCGAG
1810	1820	1830	1840	1850	1860
GGGTGCGGAG	TCTGTACACA	GCTTCTACCA	TGCTGACTGC	CACTTGGAGC	CAGTCCGTTC
1870	1880	1890	1900	1910	1920
CCAGGCACCC	CCTCCCAGAT	GCCCATCGGA	GGCATCTGGT	AGGACTGTGG	GTAGTGGGAA
1930	1940	1950	1960	1970	1980
GGTGTACCCC	ACTGTGCATA	CCAGCCCTCC	ACCAGAGATA	CTGAAGGATA	AAGCACTAGT
1990	2000	2010	2020	2030	2040
GGAGGTGGCC	CCCAGCCCTG	GGCCCCCCAC	CCTCACCAGC	TTCAACATCC	CACCTGGGCC
2050	2060	2070	2080	2090	2100
CTTCAGCTCC	ATGCACAAGC	TCCTGGAGAC	ACAGAGTACG	GGAGCCTGCC	ATAGCTCCTG
2110	2120	2130	2140	2150	2160
CAAAATCTCC	AGCCCTTGCT	CCAAGGCAGA	CAGTGGAGCC	TGCGGGCCGG	ACAGTGTCTC
2170	2180	2190	2200	2210	2220
CTACTGTGCC	CGGACAGGAG	CAGGAGAGCC	AGAGTCCGCT	GACCATGTCA	TGCCTGACTC
2230	2240	2250	2260	2270	2280
AGACAGCGAG	GCTGTGTATG	AGTTCACACA	GGACGCTCAG	CACAGTGACC	TCCGGGATCC
2290	2300	2310	2320	2330	2340
CCACAGCCGG	CGGCGACAGC	GGAGCCTGGG	CCCAGATGCA	GAGCCTAGTT	CTGTGCTGGC
2350	2360	2370	2380	2390	2400
TTTCTGGAGG	CTGATCTGTG	ACACATTCGG	GAAGATCGTA	GATAGCAAAT	ACTTTGGCCG
2410	2420	2430	2440	2450	2460
GGGAATCATG	ATCGCCATCC	TGGTCAATAC	ACTCAGCATG	GGCATCGAGT	ACCACGAGCA
2470	2480	2490	2500	2510	2520
GCCCCAGGAG	CTCACCAACG	CCCTGGAAAT	CAGCAACATC	GTCTTCACCA	GCCTCTTCGC

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2530	2540	2550	2560	2570	2580
CTTGGAGATG	CTGCTGAAAC	TGCTTGTCTA	CGGTCCCTTT	GGCTACATTA	AGAATCCCTA
2590	2600	2610	2620	2630	2640
CAACATCTTT	GATGGTGTCA	TTGTGGTTCAT	CAGTGTGTGG	GAGATTGTGG	GCCAGCAGGG
2650	2660	2670	2680	2690	2700
AGGTGGCCTG	TCGGTGCTGC	GGACCTTCCG	CCTGATGCGG	GTGCTGAAGC	TGGTGGCCTT
2710	2720	2730	2740	2750	2760
CCTGCCGGCC	CTGCAGCGCC	AGCTCGTGGT	GCTCATGAAG	ACCATGGACA	ACGTGGCCAC
2770	2780	2790	2800	2810	2820
CTTCCTCATG	CTCCTCATGC	TGTTCATCTT	CATCTTCAGC	ATCCTGGGCA	TGCACTCTCTT
2830	2840	2850	2860	2870	2880
TGGTTGCAAG	TTCGCATCTG	AACGGGATGG	GGACACGTTG	CCAGACCGGA	AGAATTTCGA
2890	2900	2910	2920	2930	2940
CTCCCTGCTC	TGGGCCATCG	TCACTGTCTT	TCAGATTCTG	ACTCAGGAAG	ACTGGAATAA
2950	2960	2970	2980	2990	3000
AGTCCTCTAC	AACGGCATGG	CCTCCACATC	GTCTTGGGCT	GCTCTTTACT	TCATCGCCCT
3010	3020	3030	3040	3050	3060
CATGACTTTT	GGCAACTATG	TGCTCTTTAA	CCTGCTGGTG	GCCATCTCTG	TGGAAGGATT
3070	3080	3090	3100	3110	3120
CCAGGCAGAG	GAAATCGGCA	AACGGGAAGA	TGGGAGTGGA	CAGTTAAGCT	GTAATCAGCT
3130	3140	3150	3160	3170	3180
GCCTGTCAAC	TCTCAGGGGG	GAGATGCCAC	CAAGTCTGAG	TCAGAGCCTG	ATTTCTTTTC
3190	3200	3210	3220	3230	3240
GCCCAGTGTG	GATGGTGATG	GGGACAGAAA	GAAGCGCTTG	GCCCTGGTGG	CTTTGGGAGA
3250	3260	3270	3280	3290	3300
ACACGGCGAA	CTACGAAAGA	GCCTTTTGCC	ACCCCTCATC	ATCCATACGG	CTGCGACACC
3310	3320	3330	3340	3350	3360
AATGTCACCTA	CCCAAGAGCT	CCAGCACAGG	TGTGGGGGAA	GCACTGGGCT	CTGGCTCTCG
3370	3380	3390	3400	3410	3420
ACGTACCAGT	AGCAGTGGGT	CCGCTGAGCC	TGGAGCTGCC	CACCATGAGA	TGAAATCTCC
3430	3440	3450	3460	3470	3480
GCCAAGTGCC	CGCAGCTCCC	CGCACAGTCC	CTGGAGTGCG	GCAAGCAGCT	GGACCAGCAG
3490	3500	3510	3520	3530	3540
GCGCTCCAGC	AGGAACAGCC	TGGGCCGGGC	CCCCAGCCTA	AAGCGGAGGA	GCCCCAGCGG
3550	3560	3570	3580	3590	3600
GGAGCGGAGG	TCCCTGCTGT	CTGGAGAGGG	CCAGGAGAGT	CAGGATGAGG	AGGAAAGTTC
3610	3620	3630	3640	3650	3660
AGAAGAGGAC	CGGGCCAGCC	CAGCAGGCAG	TGACCATCGC	CACAGGGGTT	CCTTGGAAACG
3670	3680	3690	3700	3710	3720
TGAGGCCAAG	AGTTCTCTTG	ACCTGCCTGA	CAGTCTGCAG	GTGCCGGGGC	TGCACCGCAC
3730	3740	3750	3760	3770	3780
AGCCAGCGGC	CGGAGCTCTG	CCTCTGAGCA	CCAAGACTGT	AATGGCAAGT	CGGCTTCAGG

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3790	3800	3810	3820	3830	3840
GCGTTTGGCC	CGCACCTGGA	GGACTGATGA	CCCCCAACTG	GATGGGGATG	ATGACAATGA
3850	3860	3870	3880	3890	3900
TGAGGGAAAT	CTGAGCAAAG	GGGAACGCAT	ACAAGCCTGG	GTCAGATCCC	GGCTTCCTGC
3910	3920	3930	3940	3950	3960
CTGTTGCCGA	GAGCGAGATT	CCTGGTCCGC	CTATATCTTT	CCTCCTCAGT	CAAGGTTTCG
3970	3980	3990	4000	4010	4020
TCTCCTGTGT	CACCGGATCA	TCACCCACAA	GATGTTTGAC	CATGTGGTCC	TCGTCAATCAT
4030	4040	4050	4060	4070	4080
CTTCCTCAAC	TGTATCACCA	TCGCTATGGA	GCGCCCCAAA	ATTGACCCCC	ACAGCGCTGA
4090	4100	4110	4120	4130	4140
GCGCATCTTC	CTGACCTCT	CCAACTACAT	CTTCACGGCA	GTCTTCTAG	CTGAAATGAC
4150	4160	4170	4180	4190	4200
AGTGAAGGTG	GTGGCACTGG	GCTGGTGCTT	TGGGGAGCAG	GCCTACCTGC	GCAGCAGCTG
4210	4220	4230	4240	4250	4260
GAATGTGCTG	GACGGCTTGC	TGGTGCTCAT	CTCCGTCAATC	GACATCCTGG	TCTCCATGGT
4270	4280	4290	4300	4310	4320
CTCCGACAGC	GGCACCAAGA	TCCTTGGCAT	GCTGAGGGTG	CTGCGGCTGC	TGCGGACCCT
4330	4340	4350	4360	4370	4380
GCGTCCACTC	AGGGTCATCA	GCCGGGGCCA	GGGACTGAAG	CTGGTGGTAG	AGACTCTGAT
4390	4400	4410	4420	4430	4440
GTCATCCCTC	AAACCCATTG	GCAACATGT	GGTCATTTC	TGTGCCTTCT	TCATCATTTT
4450	4460	4470	4480	4490	4500
TGGAATTCTC	GGGGTGCAGC	TCTTCAAAGG	GAAGTTCTTC	GTGTGTCAAG	GTGAGGACAC
4510	4520	4530	4540	4550	4560
CAGGAACATC	ACTAACAAAT	CCGACTGCGC	TGAGGCCAGC	TACCGATGGG	TCCGGCACAA
4570	4580	4590	4600	4610	4620
GTACAACTTT	GACAACCTGG	GCCAGGCTCT	GATGTCCCTG	TTTGTGCTGG	CCTCCAAGGA
4630	4640	4650	4660	4670	4680
TGGTGGGTT	GACATCATGT	ATGATGGGCT	GGATGCTGTG	GGTGTGGATC	AGCAGCCCAT
4690	4700	4710	4720	4730	4740
CATGAACCAC	AACCCCTGGA	TGCTGCTATA	CTTCATCTCC	TTCTCTCTCA	TCGTGGCCTT
4750	4760	4770	4780	4790	4800
CTTTGTCTTG	AACATGTTTG	TGGGCGTGGT	GGTGGAGAAC	TTCCATAAGT	GCAGACAGCA
4810	4820	4830	4840	4850	4860
CCAGGAGGAG	GAGGAGGCGA	GGCGGCGTGA	GGAGAAGCGA	CTACGGAGGC	TGGAGAAAAA
4870	4880	4890	4900	4910	4920
GAGAAGGAAT	CTAATGTTGG	ACGATGTAAT	TGCTTCCGGC	AGCTCAGCCA	GCGCTGCGTC
4930	4940	4950	4960	4970	4980
AGAAGCCCAG	TGCAAGCCCT	ACTACTCTGA	CTACTCGAGA	TTCCGGCTCC	TTGTCCACCA
4990	5000	5010	5020	5030	5040
CCTGTGTACC	AGCCACTACC	TGGACCTCTT	CATCACTGGT	GTCATCGGGC	TGAACGTGGT

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CACTATGGCC	ATGGAACATT	ACCAGCAGCC	CCAGATCCTG	GACGAGGCTC	TGAAGATCTG
5110	5120	5130	5140	5150	5160
CAATTACATC	TTTACCGTCA	TCTTTGTCTT	TGAGTCAGTT	TTCAAACCTTG	TGGCCTTTGG
5170	5180	5190	5200	5210	5220
CTTCCGCCGT	TTCTTCCAGG	ACAGGTGGA	CCAGCTGGAC	CTGGCTATTG	TGCTTCTGTC
5230	5240	5250	5260	5270	5280
CATCATGGGC	ATCACACTGG	AGGAGATTGA	GGTCAATGCT	TGGCTGCCCA	TCAACCCAC
5290	5300	5310	5320	5330	5340
CATCATCCGT	ATCATGAGGG	TGCTCCGCAT	TGCTCGAGTT	CTGAAGCTGT	TGAAGATGGC
5350	5360	5370	5380	5390	5400
TGTGGGCATG	CGGGCACTGC	TGGACACGGT	GATGCAGGCC	CTGCCCCAGG	TGGGGAACCT
5410	5420	5430	5440	5450	5460
GGGACTTCTC	TTTATGTTAT	TGTTTTTCAT	CTTTGCAGCT	CTGGGGCTGG	AGCTCTTTGG
5470	5480	5490	5500	5510	5520
AGACCTGGAG	TGTGATGAGA	CACACCCTTG	TGAGGGCTTG	GGTCGGCATG	CCACCTTTAG
5530	5540	5550	5560	5570	5580
GAACCTTTGGT	ATGGCCTTTC	TGACCCICTT	CCGAGTCTCC	ACTGGTGACA	ACTGGGAATGG
5590	5600	5610	5620	5630	5640
TATTATGAAG	GACACCCTCC	GGGACTGTGA	CCAGGAGTCC	ACCTGCTACA	ACACTGTTCAT
5650	5660	5670	5680	5690	5700
CTCCCCATAC	TACTTTGTGT	CCTTCGTGCT	GACGGCCCAG	TTTGTGCTGG	TCAACGTGGT
5710	5720	5730	5740	5750	5760
CATAGCTGTG	CTGATGAAGC	ACCTGGAAGA	AAGCAACAAA	GAGGCCAAGG	AGGAGGCCGA
5770	5780	5790	5800	5810	5820
GCTCGAGGCC	GAGCTGGAGC	TGGAGATGAA	GACGCTCAGC	CCGACGCCCC	ACTCCCCGCT
5830	5840	5850	5860	5870	5880
GGGCAGCCCC	TTCTCTCTGC	CCGGGGTGG	GGGTGTCAAC	AGTCCTGACA	GCCCTAAGCC
5890	5900	5910	5920	5930	5940
TGGGGCTCCA	CACACCACTG	CCCACATTGG	AGCAGCCTCG	GGCTTCTCCC	TTGAGCACCC
5950	5960	5970	5980	5990	6000
CACGATGGTA	CCCCACCCCG	AGGAGGTGCC	AGTCCCCCTA	GGACCAGACC	TGCTGACTGT
6010	6020	6030	6040	6050	6060
GAGGAAGTCT	GGTGTCAGCC	GGACGCACTC	TCTGCCCCAT	GACAGCTACA	TGTGCCGCAA
6070	6080	6090	6100	6110	6120
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6130	6140	6150	6160	6170	6180
AGGCTCCATC	TTGTCCGTTT	ACTCCCAACC	AGCAGACACC	AGCTGCATCC	TACAGCTTCC
6190	6200	6210	6220	6230	6240
CAAAGATGTG	CACTATCTGC	TCCAGCCTCA	TGGGGCCCCC	ACCTGGGGCG	CCATCCCTAA
6250	6260	6270	6280	6290	6300
ACTACCCCCA	CCTGGCCGCT	CCCTCTTGGC	TCAGAGGCCT	CTCAGGCCCC	AGGCAGCAAT

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6370	6380	6390	6400	6410	6420
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GGTGCAGCAG	CGTTCGGGCA	TCCAGAGCAA	AGTCTCCAAG	CACATCCGCC	TGCCAGCCCC
6490	6500	6510	6520	6530	6540
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6550	6560	6570	6580	6590	6600
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6610	6620	6630	6640	6650	6660
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6670	6680	6690	6700	6710	6720
GCCTGGGTCC	TGGCTAGATG	AACAGCGGAG	ACACTCCATT	GCTGTGAGCT	GTCTGGACAG
6730	6740	6750	6760	6770	6780
CGGCTCCCAA	CCCCGCCCTAT	GTCCAAGCCC	CTCAAGCCTC	GGGGGCCAAC	CTCTTGGGGG
6790	6800	6810	6820	6830	6840
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6970	6980	6990	7000	7010	7020
CCCAAGAAAA	GACACGCTGA	GTCTCTCTGG	TTTGTCTTCT	GACCCAACAG	ACATGGACCC
7030	7040	7050	7060	7070	7080
CTGAGTCCTA	CCCACTCTCC	CCCATCACCT	TTCTCCACCG	GGTGCAGATC	CTAGCTCCGC
7090	7100	7110	7120	7130	7140
CTCCTGGGCA	GCGTTTCTGA	AAAGTCCCCAC	GTAAGCAGCA	AGCAGCCACG	AGGCACCTCA
7150	7160	7170	7180	7190	7200
CCTGCCCTTCT	TCAGTGGCTG	GTGGGGATGA	CGAGCAGAAC	TTCCGGAGAG	TCGATCTGAA
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GAGAACACAG	CCCTGGAGCC	CCTGCCCTCCG	GGAAGAAGGA	AAAGGAGAAA	GCCCAGTGTG
7270	7280	7290	7300	7310	7320
GCCAAGGCTC	CCGACACCAG	GAGCTG

end of
seq 1 + 3

SEQ 19 No: 4

start of
SEQ 19 No: 2
(coding region)

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 RVSMVLVILLNCVTLGMFRPCEDIACDSQRCRILQAFDDFIFAFV
 EMVVKMVALGIFGKKCYLGDTWNRLDFFIVIAGMLEYSDDLQNV
 FSAVRTVRVLRPLRAINRVPSMRILVTLTLLDTLPLMLGNVLLCFFV
 FFIFGIVGVQLWAGLLRNRCFLPENFSLPLSVDEPYQTENEDES
 PFICSQPRENGMRSCRSVPTLRGEGGGGPPCSLDYETYNSSSNTT
 CVNWNQYYTNC SAGEHNPFKGAINFDNIGYAWIAIFQVITLEGWV
 DIMYFVMDAHSFYNFYIFILLIIVGSFFMINLCLVVIATQFSETKQR
 ESQLMREQRVRFLSNASTLASFSEPGSCYEELLKYLVYILRKAAR
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 VHHHHHHHHHHYHLGNGTLRVPRASPEIQDRDANGSRRLMLPPPST
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 MHKLLETQSTGACHSSCKISSPCSKADSGACGPDSCPYCARTGAG
 EPESADHVMPSDSEAVYEFTQDAQHSDLRDPHSRRRQRSLGPD
 EPSSVLAFWRLICDTFRKIVDSKYFGRGIMIAILVNTLSMGIEYHEQ
 PEELTNALEISNIVFTSLFALEMLLKLVLVYGPFGYIKNPYNIFDGI
 VVISVWEIVGQQGGGLSVLRTFRLMRVLKLVRFPLALQRLVVL
 KTMDNVATFCMLLMFLFIFISILGMHLFGCKFASERDGD TLPDR
 NFD SLLWAI VTFVQILTQEDWNKVLVNGMASTSSWAALYFIALMT
 FGNYVLFNLLVAILVEGFQAEI GKREDASGQLSCIQLPVNSQGGD
 ATKSESEP DFFSPSV DGDGDRKKRLALVALGEHAELRKSLLPPLII
 HTAATPMSLPKSSSTGVGEALGSGSRRTSSSGSAEPGAHHHEKMS
 PPSARSSPHSPWSAASSWTSRRSSRNSLGRAPSLKRRSPSGERRS
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 TLQVPGLHRTASGRSSASEHQDCNGKSASGRLARTLRDTPQLDG
 DDDNDEGNLSKGERIQAWVRSRLPACCRERDSWSAYIFPPQSRFR
 LLCHRIITHKMFHDHVVLVIFLNCITIAMERPKIDPHSAERIFLTLSN
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 DILVSMVSDSGTKILGMLRVLRLRLRTRPLRVLISRAQGLKLVVETL
 MSSLKPIGNIVVICCAFFIIFGILGVQLFKGKFFVCQGEDTRNITNK
 SDCAEAS YRWVRHKYNFDNLGQALMSLFLVASKDGWVDIMYDGL
 DAVGVDQQPIMNHNFWMLLYFISFLLIVAFFVLNMFVGVVVENFH
 KCRQH QEEEEARRREEKRLRRLEKKRRNLMLDDV IASGSSASAAS
 EAQCKPYYS DYSRFRLLVHHLCTSHYLDL FITGVIGLVVVTMAME
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 AVGMRALLDTVMQALPQVGNLGLL FMLLFFIFAALGVELFGDLEC
 DETHPCEGLGRHATFRNFGMAFLTFRVSTGDNWNGIMKDTLRDC
 DQESTCYNTVISPIYFVSFVLTAQFVLVNVVIAVLMKHLEESNKEA
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 TSCILQLPKDVHYLLQPHGAPTWGAIPKLPPPGRSPLAQRPLRQA
 AIRTDSL DVQGLGSREDLLSEVSGPSCPLTRSSSFWGGSSIQVQQR
 SGIQSKVSKHIRLPAPCPGLEPSWAKDPPETRSSL ELDTELSWISG
 DLLPSSQEEPLSPRDLKKCYSVETQSCRRRPGSWLDEQRRHSIAY
 SCLDSGSQPRLCPSPSLGGQPLGGPGSRPKKLSPPSISIDPPESQ
 GSRPPCSPGVCLRRRAPASDSKDPSVSSPLDSTAASPSPKKDTLSL
 SGLSSDPTDMDP*VLPTLPHHLSPPGADPSSASWAAFLKSPT*AAS
 SH2071EAPHLPSSVAGGDDEQNFRRVDLKRTPQWSPCLREEGKGE
 SPVWPRLPTPGA

SEQ 2 + 4

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SEQ ID NO:5: SKEKQMA

SEQ ID NO:6: 5'TNGC(A/C/T)ATGGAG(C/A)GNCC(C/T)-3'

SEQ ID NO:7: 5'-CTT(C/G/T)CCCTTGAA(G/C)A(G/A)CTG)-3'

SEQ ID NO:8: 5'-CCGCTGTCGGAGACCATGGAGACC-3'

SEQ ID NO:9: 5'-AGCGGCCCAAATTGACCCCCACAG-3' —

SEQ ID NO:10: 5'-GAAGATGCGAGTGGACAG-3'

SEQ ID NO:11: 5'-CTGTGGCGATGGTCACTG-3'

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What Is Claimed Is:

1 1. An isolated nucleic acid molecule encoding a
2 pancreatic T-type calcium channel.

1 2. The isolated nucleic acid molecule of claim 1
2 wherein said nucleic acid is deoxyribonucleic acid.

1 3. The isolated nucleic acid molecule of claim 2
2 wherein said deoxyribonucleic acid is cDNA.

1 4. The isolated nucleic acid molecule of claim 3
2 wherein said nucleic acid molecule has a nucleotide
3 sequence as shown in SEQ ID NO:1.

1 5. The isolated nucleic acid molecule of claim 1
2 wherein said nucleic acid molecule encodes an amino acid
3 sequence as shown in SEQ ID NO:2.

1 6. The isolated nucleic acid molecule of claim 1
2 wherein said nucleic acid is ribonucleic acid.

1 7. The isolated nucleic acid molecule of claim 6
2 wherein said ribonucleic acid is mRNA.

1 8. An antisense nucleic acid molecule complementary
2 to at least a portion of the mRNA of claim 7.

1 9. A cell comprising the antisense nucleic acid
2 molecule of claim 8.

1 10. An expression vector comprising the antisense
2 nucleic acid molecule of claim 8.

1 11. The expression vector of claim 10 wherein the
2 expression vector is selected from the group consisting
3 of a plasmid and a virus.

1 12. A cell comprising the expression vector of
2 claim 10.

1 13. A method of decreasing expression of a
2 pancreatic T-type calcium channel in a host cell, said
3 method comprising introducing the antisense nucleic acid
4 molecule of claim 8 into the cell, wherein said antisense
5 nucleic acid molecule blocks translation of said mRNA so
6 as to decrease expression of said pancreatic T-type
7 calcium channel in said host cell.

1 14. A ribozyme having a recognition sequence
2 complementary to a portion of the mRNA of claim 7.

1 15. A cell comprising the ribozyme of claim 14.

1 16. An expression vector comprising the ribozyme of
2 claim 14.

1 17. The expression vector of claim 16 wherein the
2 expression vector is selected from the group consisting
3 of a plasmid and a virus.

1 18. A cell comprising the expression vector of
2 claim 16.

1 19. A method of decreasing expression of a
2 pancreatic T-type calcium channel in a host cell, said
3 method comprising introducing the ribozyme of claim 14
4 into the cell, wherein expression of said ribozyme in

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5 said cell results in decreased expression of said
6 pancreatic T-type calcium channel in said cell.

1 20. A cell comprising the nucleic acid molecule of
2 claim 1.

1 21. An expression vector comprising the nucleic
2 acid molecule of claim 1.

1 22. The expression vector of claim 21 wherein said
2 expression vector is selected from the group consisting
3 of a plasmid and a virus.

1 23. A cell comprising the expression vector of
2 claim 21.

1 24. A method of increasing expression of pancreatic
2 T-type calcium channel in a host cell, said method
3 comprising:

4 introducing the nucleic acid molecule of
5 claim 1 into the cell; and

6 allowing said cell to express said nucleic acid
7 molecule resulting in the production of pancreatic T-type
8 calcium channel in said cell.

1 25. A method of screening a substance for the
2 ability of the substance to modify T-type calcium channel
3 function, said method comprising:

4 introducing the nucleic acid molecule of claim 1
5 into a host cell;

6 expressing said pancreatic T-type calcium channel
7 encoded by said nucleic acid molecule in the host cell;

8 exposing the cell to a substance; and

9 evaluating the exposed cell to determine if the
10 substance modifies the function of the T-type calcium
11 channel.

1 26. The method of claim 25 wherein said evaluation-
2 comprises monitoring the expression of T-type calcium
3 channel.

1 27. A method of obtaining DNA encoding a pancreatic
2 T-type calcium channel, said method comprising:
3 selecting a DNA molecule encoding a pancreatic T-
4 type calcium channel, said DNA molecule having a
5 nucleotide sequence as shown in SEQ ID NO:1;
6 designing an oligonucleotide probe for a pancreatic
7 T-type calcium channel based on SEQ ID NO:1;
8 probing a genomic or cDNA library of an organism
9 with the oligonucleotide probe; and
10 obtaining clones from said library that are
11 recognized by said oligonucleotide probe, so as to obtain
12 DNA encoding a pancreatic T-type calcium channel.

1 28. A method of obtaining DNA encoding a pancreatic
2 T-type calcium channel, said method comprising:
3 selecting a DNA molecule encoding a pancreatic
4 T-type calcium channel, said DNA molecule having a
5 nucleotide sequence as shown in SEQ ID NO:1;
6 designing degenerate oligonucleotide primers
7 based on SEQ ID NO:1; and
8 utilizing said oligonucleotide primers in a
9 polymerase chain reaction on a DNA sample to identify
10 homologous DNA encoding a pancreatic T-type calcium
11 channel in said sample.

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1 29. An isolated nucleic acid molecule encoding a
2 pancreatic T-type calcium channel, said nucleic acid
3 molecule encoding a first amino acid sequence having at
4 least 90% amino acid identity to a second amino acid
5 sequence, said second amino acid sequence as shown in SEQ-
6 ID NO:2.

1 30. A DNA oligomer capable of hybridizing to the
2 nucleic acid molecule of claim 1.

1 31. A method of detecting presence of a pancreatic
2 T-type calcium channel in a sample, said method
3 comprising:
4 contacting a sample with the DNA oligomer of claim
5 30, wherein said DNA oligomer hybridizes to any of said
6 pancreatic T-type calcium channel present in said sample,
7 forming a complex therewith; and
8 detecting said complex, thereby detecting presence
9 of a pancreatic T-type calcium channel in said sample.

1 32. The method of claim 31 wherein said DNA
2 oligomer is labeled with a detectable marker.

1 33. An isolated pancreatic T-type calcium channel
2 protein.

1 34. The pancreatic T-type calcium channel protein
2 of claim 33 wherein said pancreatic T-type calcium
3 channel protein is encoded by a nucleotide sequence as
4 shown in SEQ ID NO:1.

1 35. The pancreatic T-type calcium channel protein
2 of claim 33 wherein said pancreatic T-type calcium

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3 channel protein is encoded by an amino acid sequence as
4 shown in SEQ ID NO:2.

1 36. An isolated pancreatic T-type calcium channel
2 protein encoded by a first amino acid sequence having at-
3 least 90% amino acid identity to a second amino acid
4 sequence, said second amino acid sequence as shown in SEQ
5 ID NO:2.

1 37. An antibody or fragment thereof specific for
2 the pancreatic T-type calcium channel protein of claim
3 36.

1 38. The antibody of claim 37 wherein said antibody
2 comprises a monoclonal antibody.

1 39. The antibody of claim 37 wherein said antibody
2 comprises a polyclonal antibody.

1 40. A composition comprising the pancreatic T-type
2 calcium channel protein of claim 36 and a compatible
3 carrier.

1 41. A method of detecting presence of a pancreatic
2 T-type calcium channel protein in a sample, said method
3 comprising:

4 contacting a sample with the antibody or fragment
5 thereof of claim 37, wherein said antibody or fragment
6 thereof binds to any of said pancreatic T-type calcium
7 channel protein present in said sample, forming a complex
8 therewith; and

9 detecting said complex, thereby detecting presence
10 of a pancreatic T-type calcium channel protein in said
11 sample.

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1 42. The method of claim 41 wherein said antibody or
2 fragment thereof is labeled with a detectable marker.

1 43. A method of modifying insulin secretion by
2 pancreatic beta cells, the method comprising modifying
3 levels of functional T type calcium channels in the
4 pancreatic beta cells.

1 44. The method of claim 43 wherein modifying levels
2 of functional T type calcium channels comprises modifying
3 T type calcium channel gene expression in the pancreatic
4 beta cells.

1 45. The method of claim 44 wherein modifying T type
2 calcium channel gene expression comprises exposing the
3 pancreatic beta cells to a compound which modifies T type
4 calcium channel gene expression.

1 46. The method of claim 45 wherein the compound is
2 an antisense oligonucleotide targeted to the T type
3 calcium channel gene.

1 47. The method of claim 43 wherein modifying levels
2 of functional T type calcium channel comprises exposing
3 the pancreatic beta cells to an inhibitor of the
4 functional T type calcium channel.

1 48. The method of claim 43 wherein modifying levels
2 of functional T type calcium channel comprises exposing
3 the pancreatic beta cells to a compound which interferes
4 with membrane T type calcium channel formation.

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1 49. The method of claim 43 wherein the pancreatic
2 beta cells are present in a subject having type II
3 diabetes.

1 50. A method of treating type II diabetes in a
2 subject, the method comprising administering to the
3 subject an amount of a compound effective to modify
4 levels of functional T type calcium channel in the
5 pancreatic beta cells of the subject.

1 51. The method of claim 50 wherein the compound
2 modifies levels of functional T type calcium channel by
3 modifying T type calcium channel gene expression.

1 52. The method of claim 51 wherein modifying T type
2 calcium channel gene expression comprises exposing the
3 pancreatic beta cells to a compound which modifies T type
4 calcium channel gene expression.

1 53. The method of claim 52 wherein the compound is
2 an antisense oligonucleotide targeted to the T type
3 calcium channel gene.

1 54. The method of claim 50 wherein the compound is
2 an inhibitor of the functional T type calcium channel.

1 55. The method of claim 50 wherein the compound
2 interferes with membrane T type calcium channel
3 formation.

1 56. A method of modifying basal calcium levels in
2 cells, the method comprising modifying levels of
3 functional T type calcium channels in the cells.

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1 57. A method of modifying the action potential of L
2 type calcium channels in cells, the method comprising
3 modifying levels of functional T type calcium channels in
4 the cells.

1 58. A method of modifying pancreatic beta cell
2 death, the method comprising modifying levels of
3 functional T type calcium channels in the pancreatic beta
4 cells.

1 59. A method of modifying pancreatic beta cell
2 proliferation, the method comprising modifying levels of
3 functional T type calcium channels in the pancreatic beta
4 cells.

1 60. A method of modifying calcium influx through L
2 type calcium channels in cells, the method comprising
3 modifying levels of functional T type calcium channels in
4 the cells.

Fig. 1A

- (1) atgctccccaccggggtcccccggttcggtgaggacacctctctgaggggtccgctcgccctcttctg
 (2) ATGctccccaccggg - tccccg-ttcggtgaggacacctctctgaggggtccgctcgccctcttctg
- (1) gacccccggggcccccggtggccagaggATggacgaggaggagga tggagcggggcgccgaggagtcggga
 (2) gacccccggggcccccggtggccagaggatggacgaggaggagga tggagcggggcgccgaggagtcggga
- (1) cagccccgtagcttcacgcagctcaacgacctgtccggggccggggcagggggccggggg
 (2) cagccccgtagcttcacgcagctcaacgacctgtccggggcgccggggc-ggcagggggccggggg-

Fig. 1B

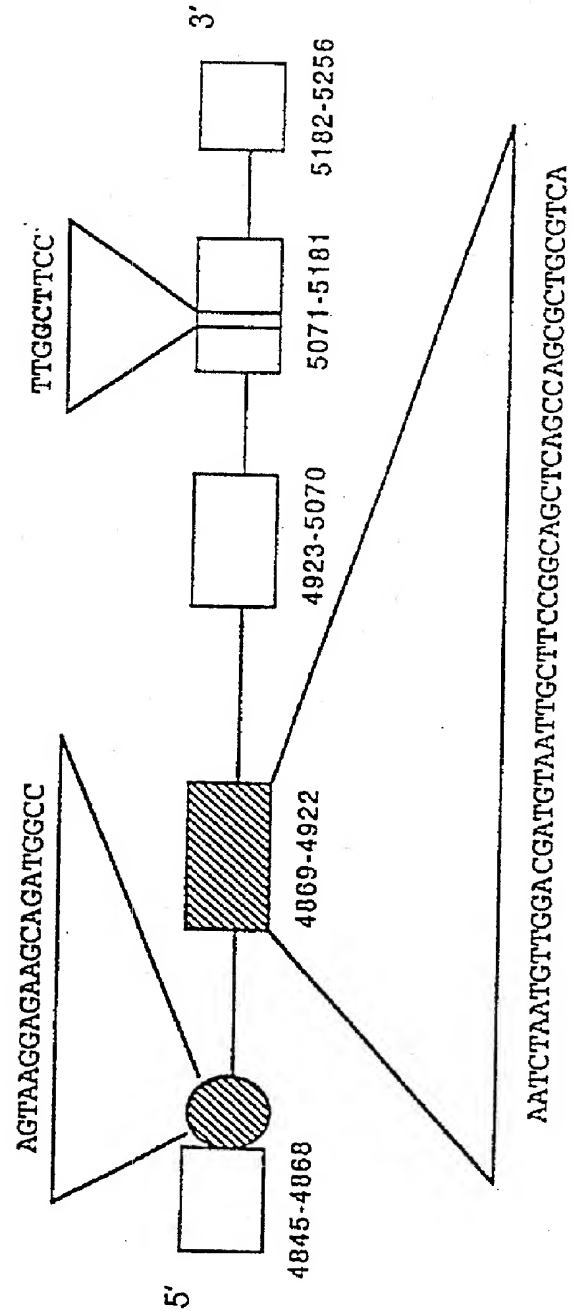


Fig. 2A

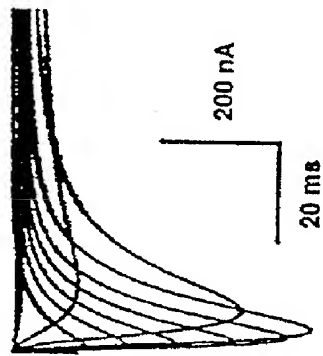


Fig. 2B

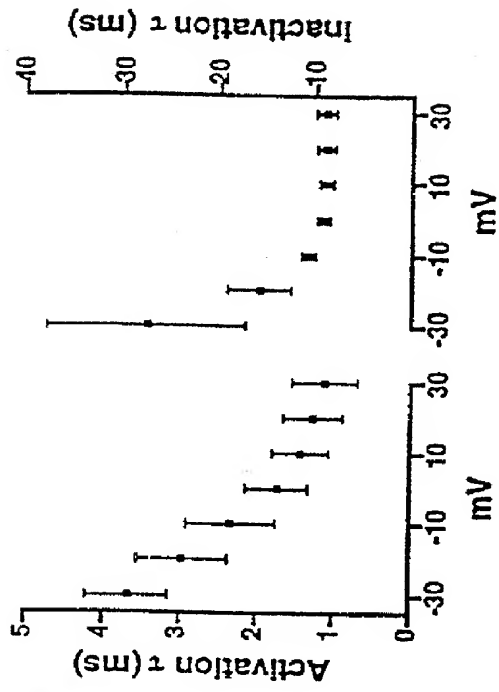


Fig. 2C

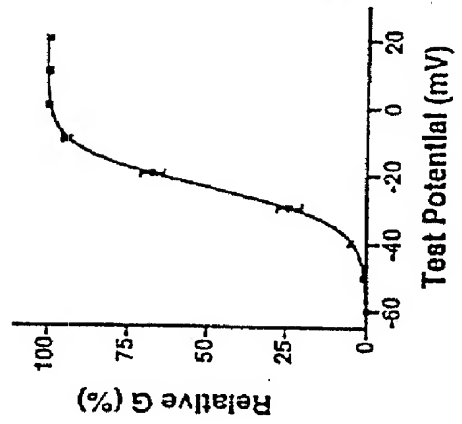


Fig. 2D

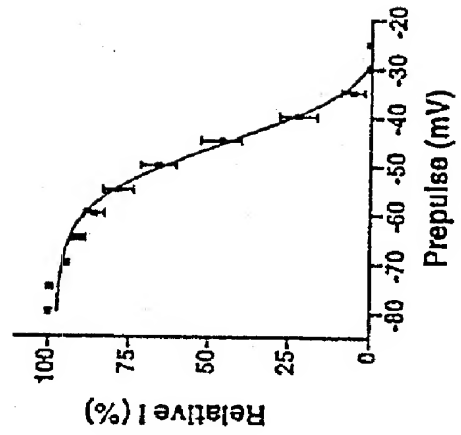


Fig. 3B

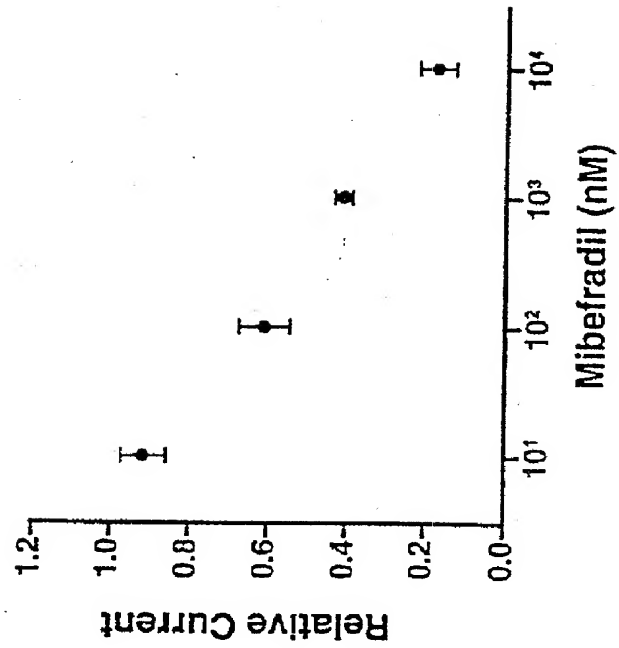


Fig. 3A

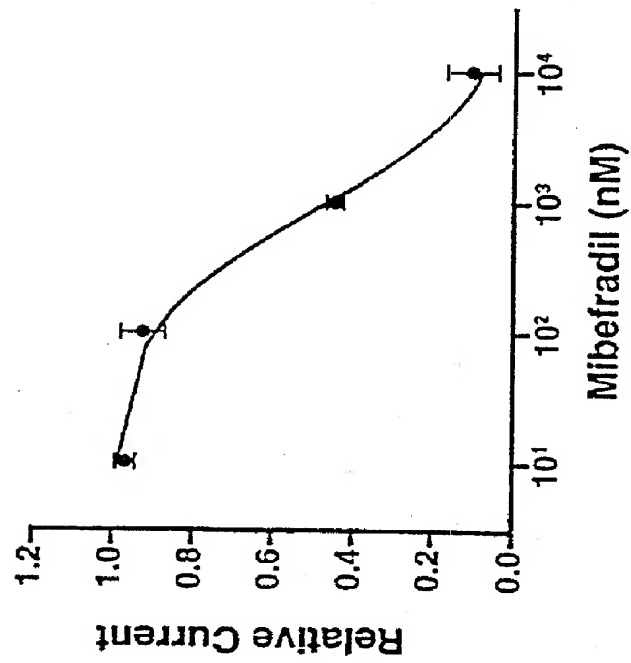


Fig. 4

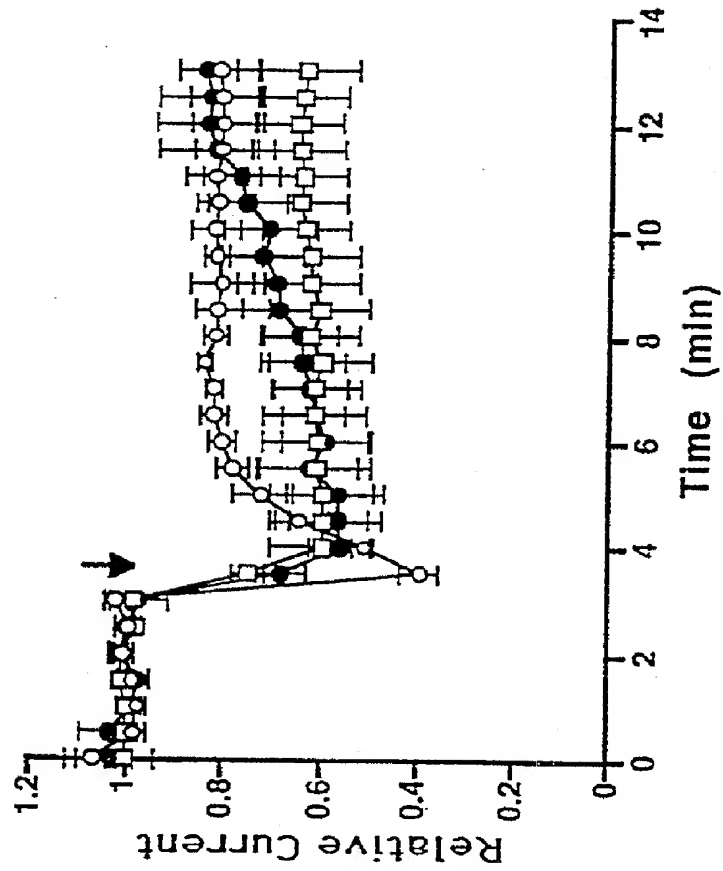


Fig. 5A

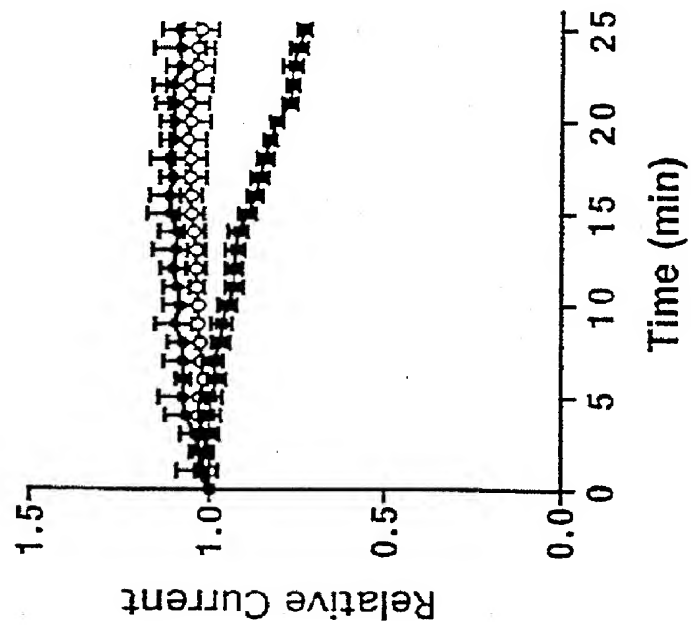
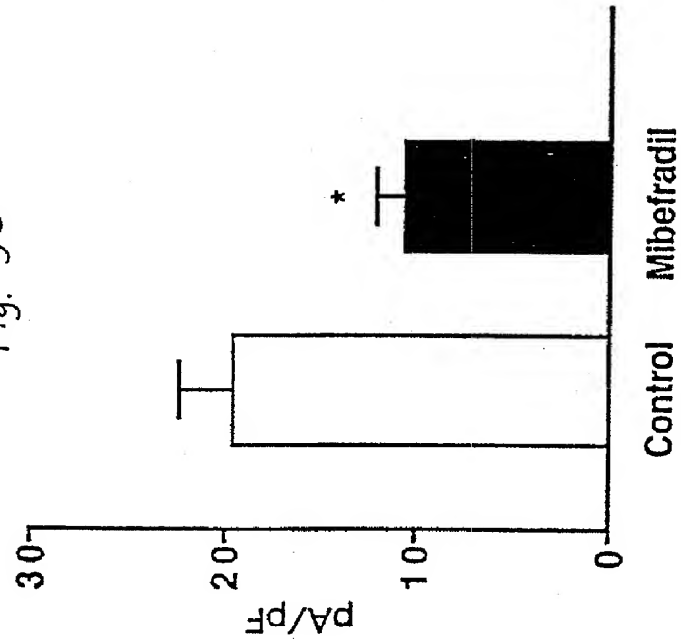


Fig. 5B



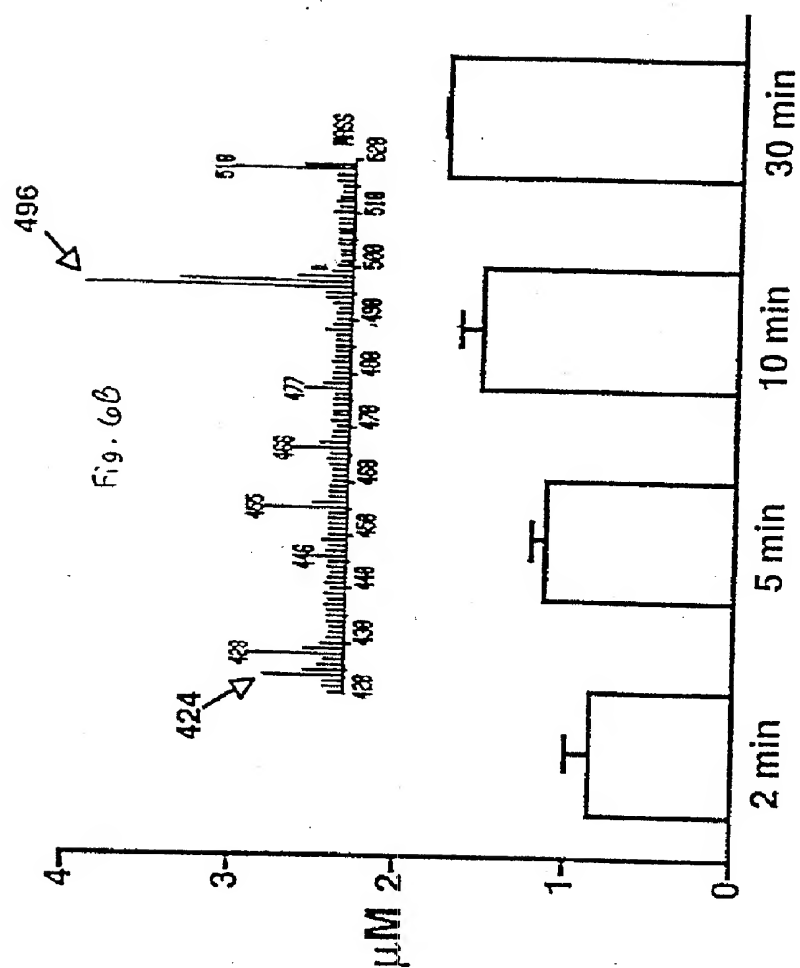


Fig. 6A

Fig. 7B

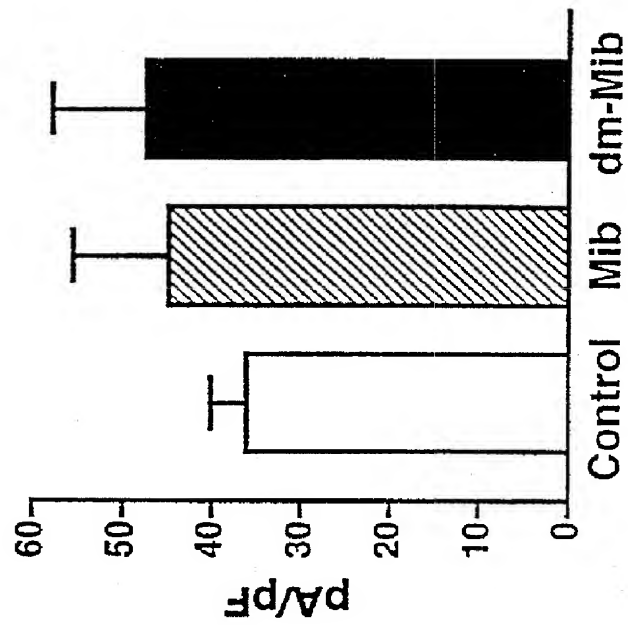
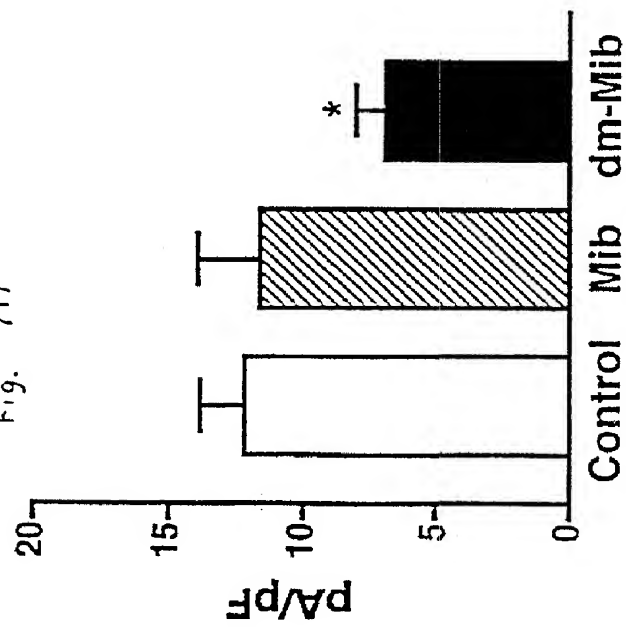


Fig. 7A



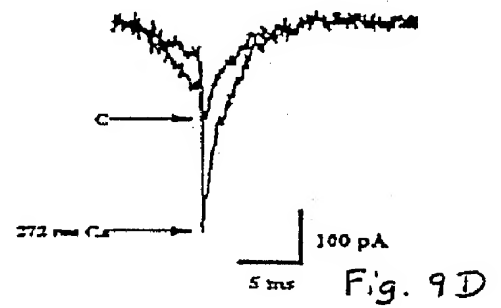
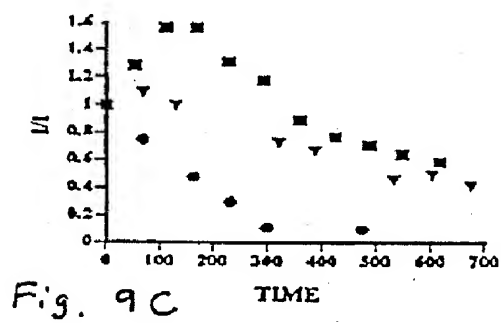
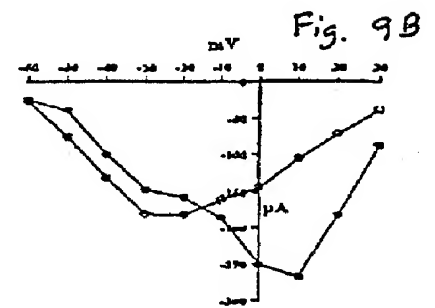
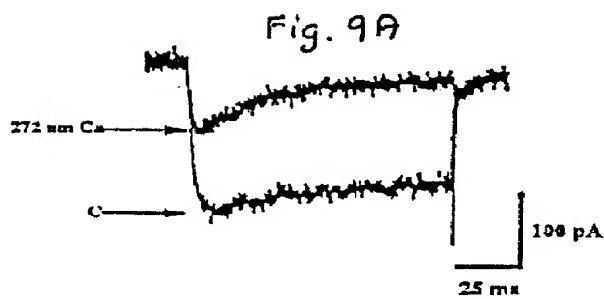
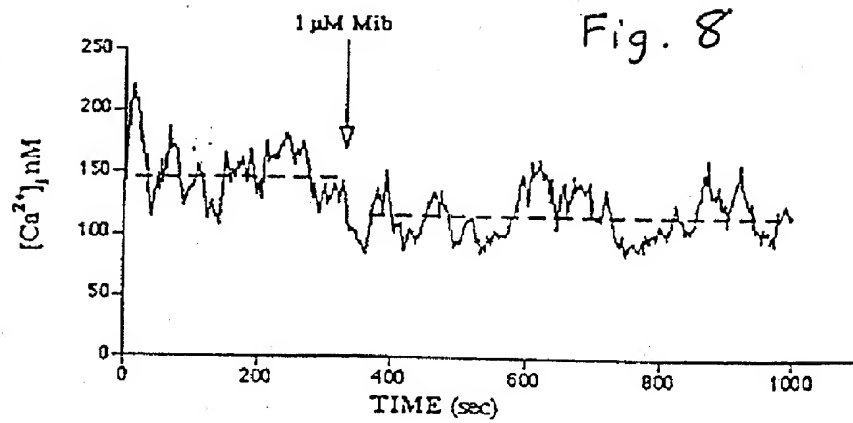


Fig. 10

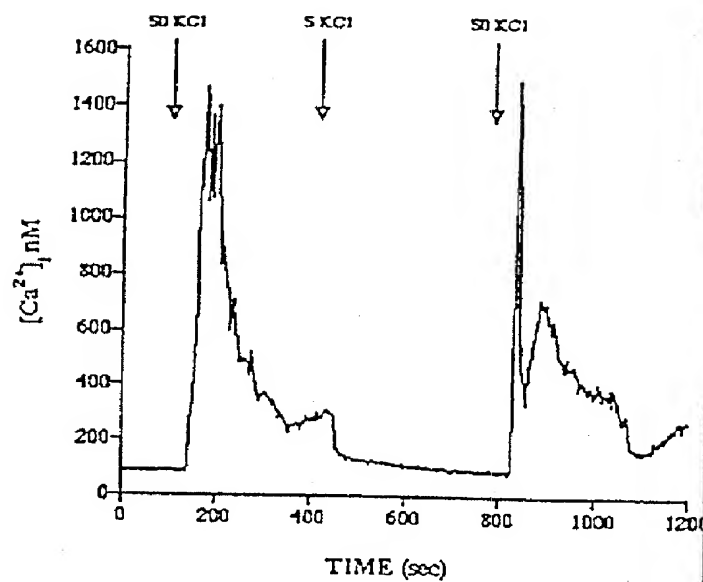


Fig. 11

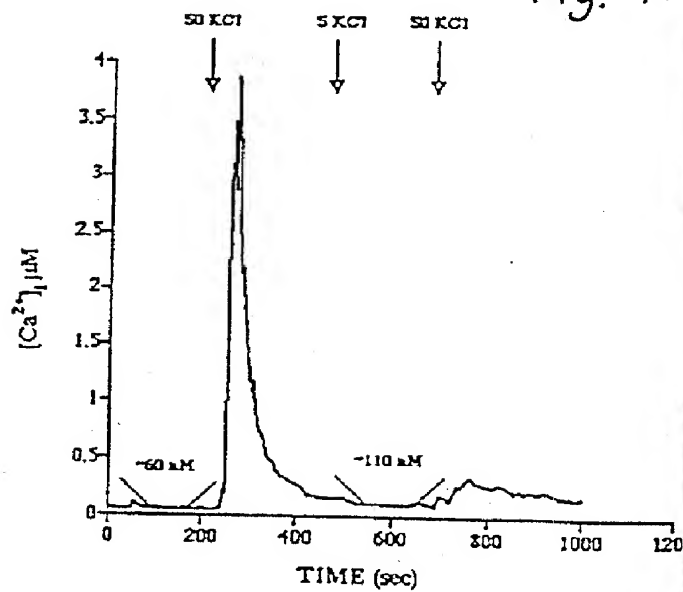


Fig. 12

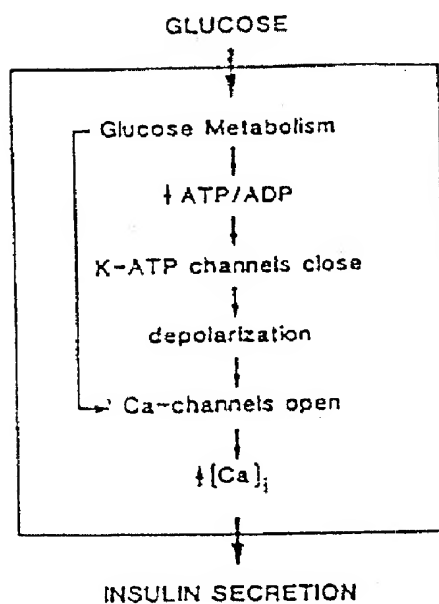
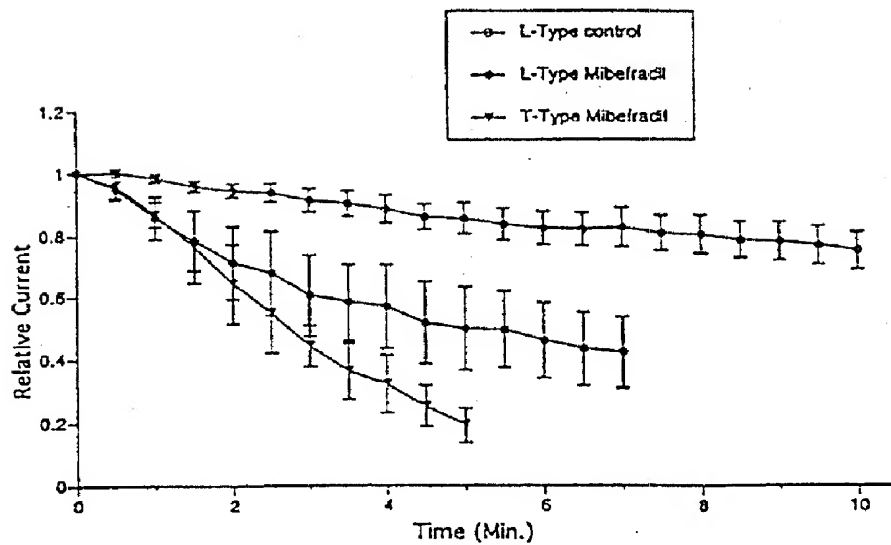


Fig. 13



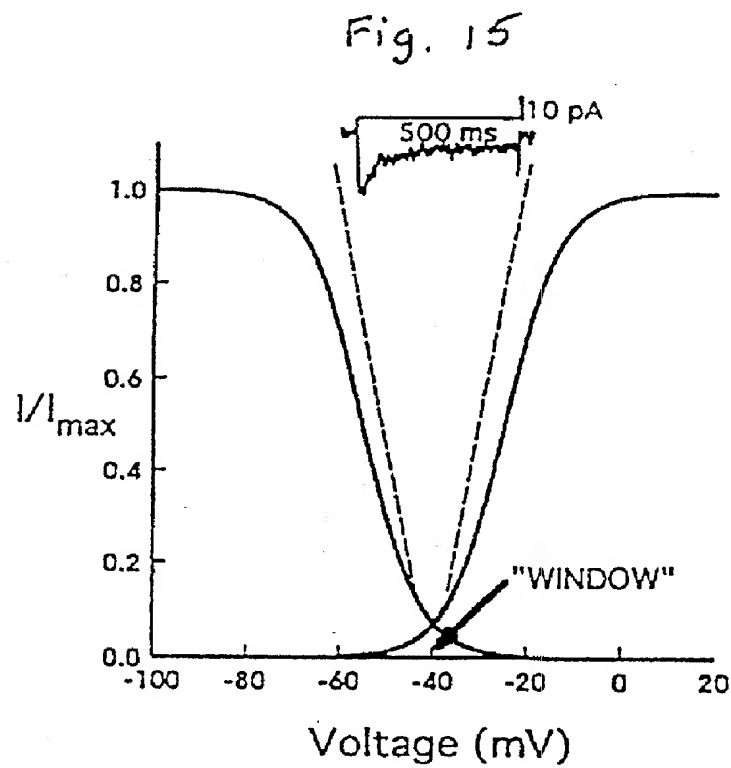
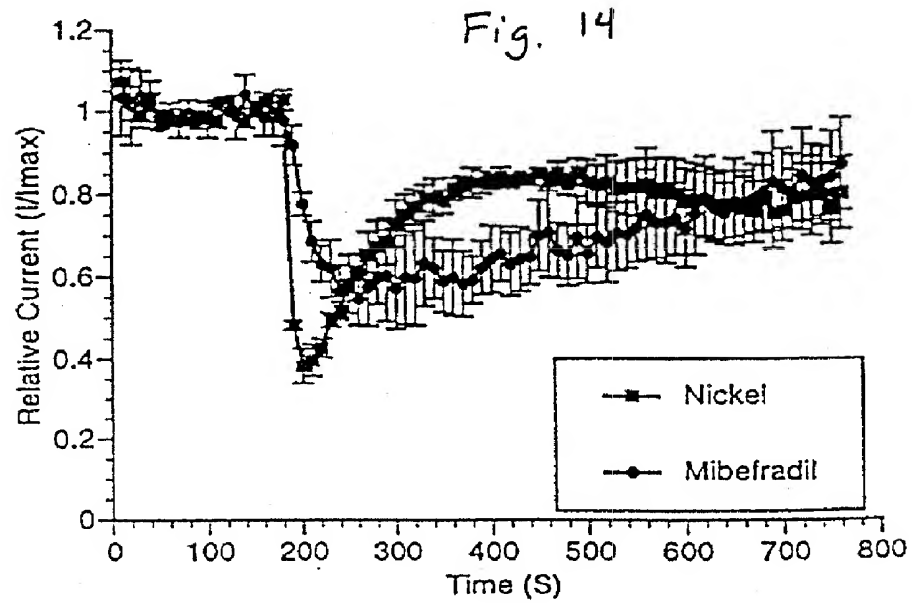


Fig. 16

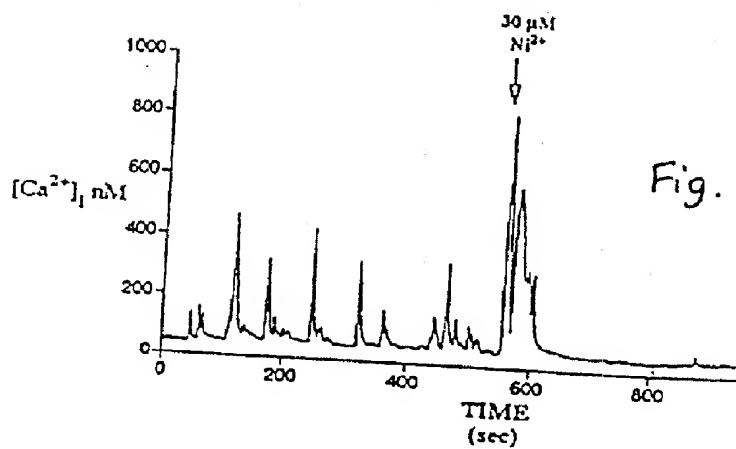
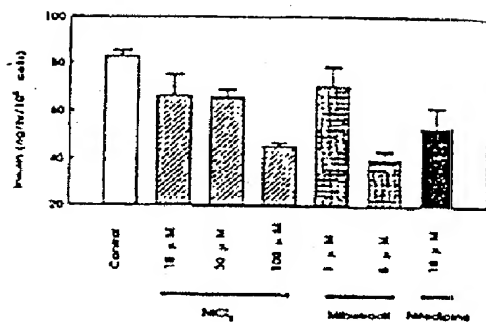
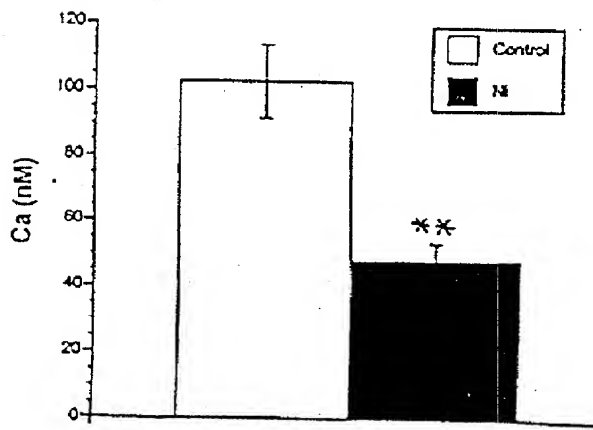


Fig. 18



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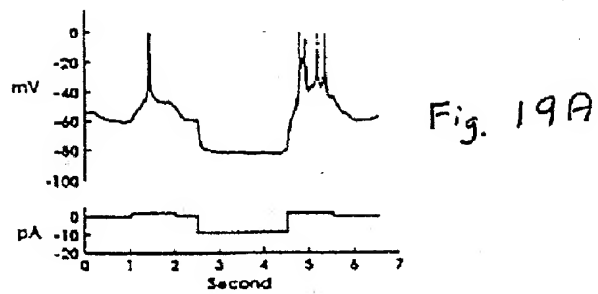


Fig. 19A

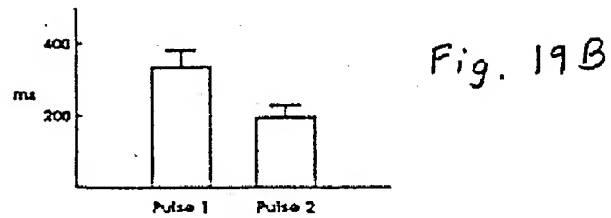


Fig. 19B

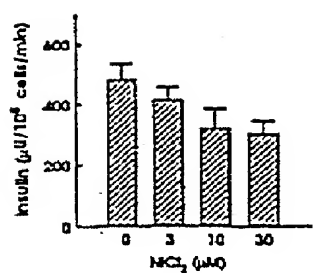


Fig. 20A

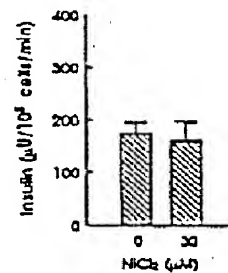
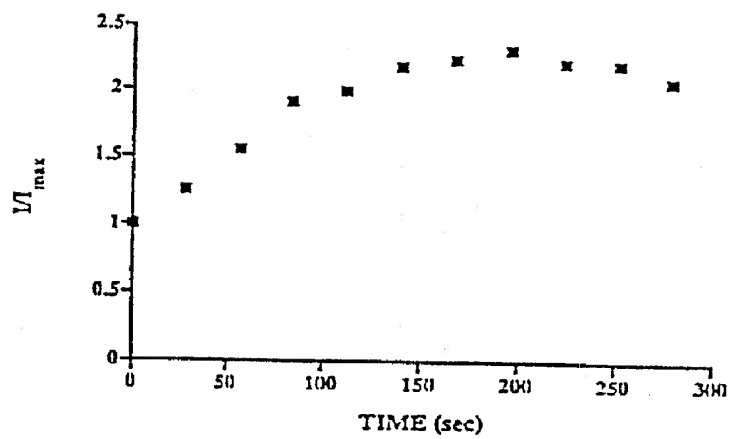


Fig. 20B

Fig. 21



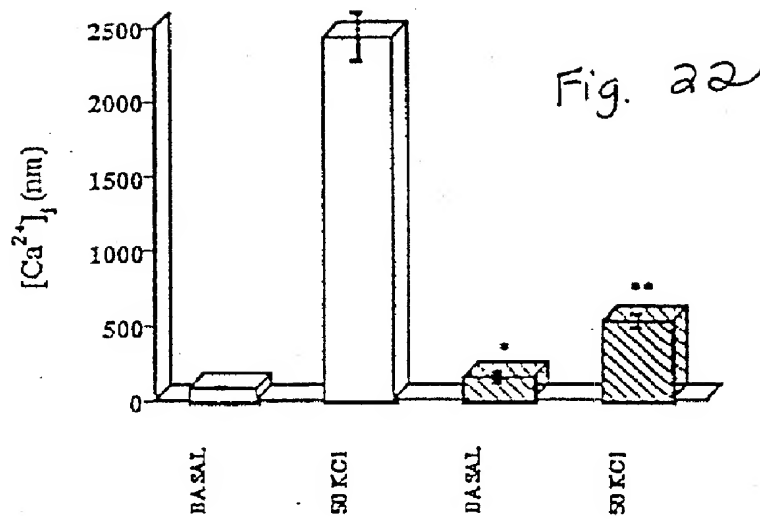


Fig. 23A.

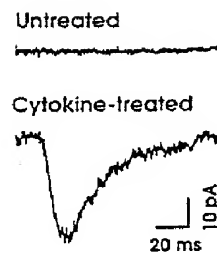


Fig. 23B.

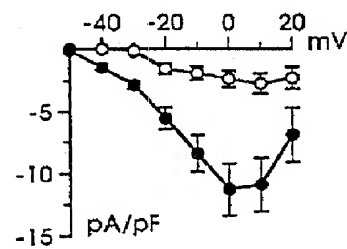


Fig. 23C.

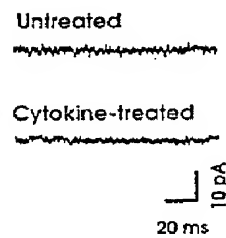


Fig. 23D.

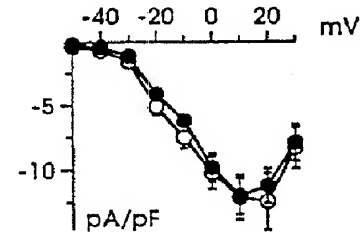


Fig. 23E.

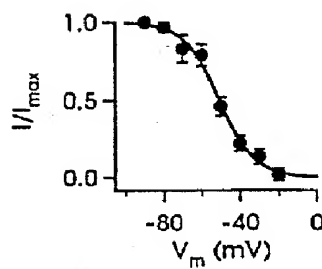


Fig. 23F.

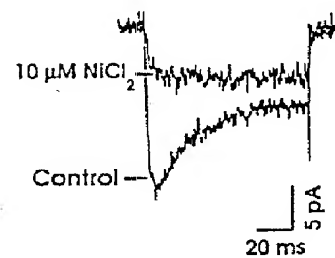


Fig. 24A.

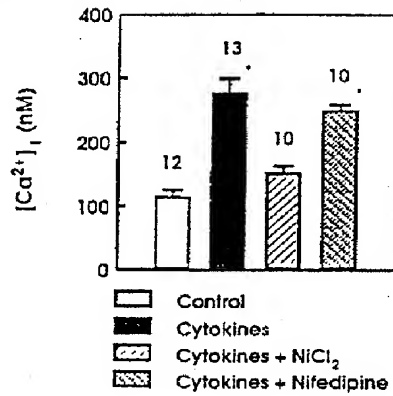


Fig. 24B.

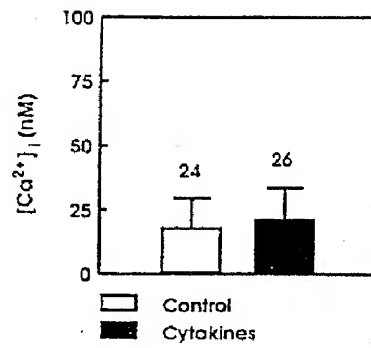


Fig. 25A.

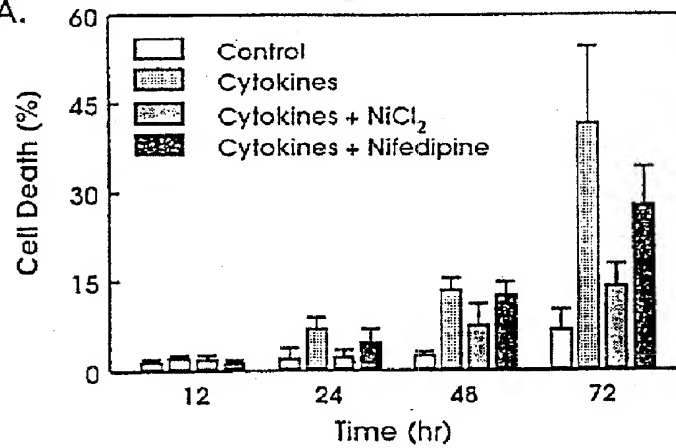


Fig. 25B.

